





National Institute of Health





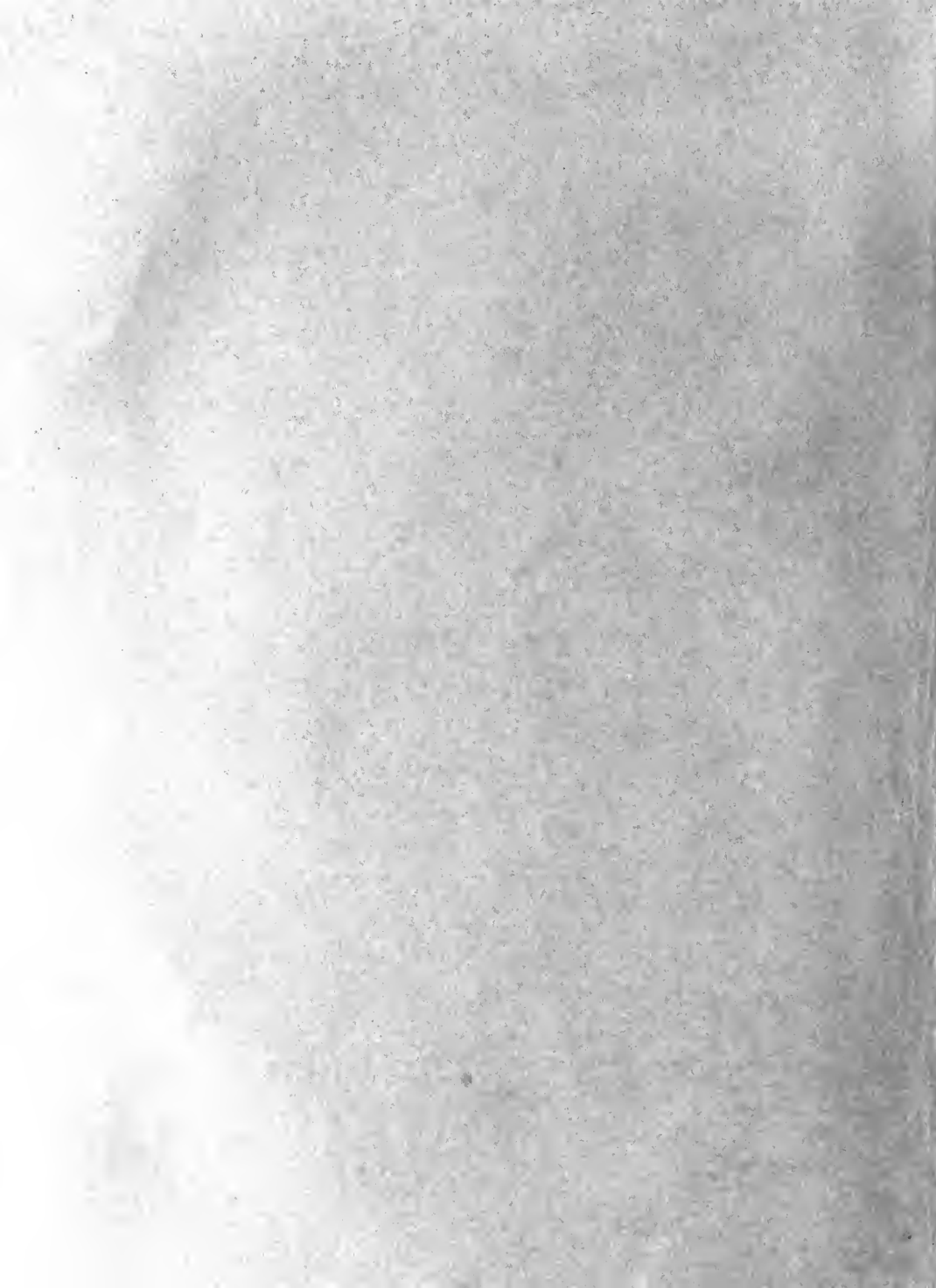




# Annual Report of Intramural Research Program Activities

National Institute on Alcohol Abuse and Alcoholism

Fiscal Year 1987



Alcohol, Drug Abuse, and  
☐ Mental Health Administration  
National Institute on  
☐ Alcohol Abuse and Alcoholism  
Intramural Research Program  
Bethesda, Maryland 20892

December 5, 1987

Attached for your information is a copy of the Annual Report  
of NIAAA Intramural Research Program Activities for fiscal year  
1987.

Boris Tabakoff



# Annual Report of Intramural Research Program Activities

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National Institute on Alcohol Abuse and Alcoholism

October 1, 1986 to September 30, 1987

Summary Statements and  
Individual Project Reports

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES  
Public Health Service  
Alcohol, Drug Abuse, and Mental Health Administration  
National Institute on Alcohol Abuse and Alcoholism  
9000 Rockville Pike  
Bethesda, Maryland 20892





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**Annual Report of the  
Division of Intramural Clinical and Biological Research  
National Institute on Alcohol Abuse and Alcoholism  
October 1, 1986 to September 30, 1987  
Director's Overview  
Boris Tabakoff, Ph.D., Director**

The National Institute on Alcohol Abuse and Alcoholism (NIAAA) Intramural Research Program (IRP) has, during this last year, continued its productive course in researching the etiologic factors of alcohol abuse and alcoholism and gaining an understanding of the processes by which alcohol produces organ damage. The IRP reorganization, which took place in FY 86, has significantly enhanced the intramural research efforts as witnessed by the vitality of the newly established programs and the successful recruiting efforts for the heads of the newly established laboratories and sections. The recruitment of Dr. Alan McLaughlin to head the Section of Physical Chemistry in the Laboratory of Metabolism and Molecular Biology has provided for the IRP a new dimension in research use of nuclear magnetic resonance (NMR) technology. The NIAAA-NMR program has been well integrated within the newly opened National Institutes of Health NMR facility, and the availability of instrumentation and expertise in the NMR area should certainly further our IRP's interests in in vivo spectroscopy of high energy phosphates, divalent cation homeostasis, and membrane biophysics. Recruitments for the Chief of the Laboratory of Physiologic and Pharmacologic Studies and the Section on Immunology, within that Laboratory, have been actively pursued during the past year, and successfully completed. Dr. George Kunos, an internationally known receptor pharmacologist, will head the Laboratory of Physiologic and Pharmacologic Studies, and Dr. Randall Kincaid, an excellent enzymologist, will manage the Section on Immunology. In addition, the IRP has focused its efforts during the last year on enhanced use of molecular genetic techniques in clinical and preclinical studies within the program. In the realm of clinical studies, major efforts are underway to generate an appropriate panel of Y chromosome probes, as well as probes of other chromosomes of the human genome. The IRP, Laboratory of Clinical Studies, is planning to utilize such probes in genetic linkage studies. Restriction fragment linked polymorphisms will be assessed in the families of alcoholics recruited for participation in our research protocols. It is hoped that such studies will provide insights into factors responsible for predisposing individuals to developing alcohol-related problems. In another set of studies using molecular genetics techniques, researchers in the Laboratory of Metabolism and Molecular Biology, have been concentrating on cloning the various isozymes of the microsomal P450 drug metabolizing systems. Prior studies have shown that alcohol ingestion induces a form of these microsomal enzymes that not only metabolizes ethanol, but also

has the propensity to transform various exogenous compounds into carcinogens. The assessment of the structure and regulation of transcription of the P450 isozymes should produce insights into alcohol's ability to act as a cofactor in the development of certain forms of cancer. The mechanisms by which metabolic tolerance to alcohol develops in some individuals will also be amenable to investigation in such studies. Molecular genetics techniques have also been applied to an analysis of chromosomal material responsible for alcohol preference in certain strains of mice. Researchers in the Laboratory of Clinical Studies have collaborated with researchers at the University of Oregon at Portland to demonstrate that the preference for alcohol in mice is determined by genetic material existing on chromosome one of the mouse genome. Such studies are the beginnings of efforts to unravel, on a molecular level, the factors responsible for alcohol intake in animals, and by this means hopefully provide a knowledge base for similar approaches to be used in studies with humans.

In addition to the above-mentioned studies using molecular genetics techniques, three other IRP areas of research deserve some special mention. The first of these areas relates to studies of pharmacologic agents which may be effective in treating the alcohol-induced amnestic syndrome evident in a substantial number of alcoholics. Intramural scientists have used the drug, fluvoxamine to obtain significant improvement in cognitive functioning in patients diagnosed as having the alcohol-induced amnestic syndrome.

Substantial efforts have also been expended in examining the factors responsible for pharmacodynamic alcohol tolerance because of the postulated relation between the development of alcohol tolerance and increased intake of alcohol in individuals. Researchers in the Laboratory of Physiologic and Pharmacologic Studies have demonstrated that the neuropeptide, vasopressin, and its analogs, can be used to modulate alcohol tolerance and, in studies with animals, vasopressin  $V_1$  receptors in brain have been shown to be the sites through which alcohol tolerance development can be controlled. Thus, knowledge being developed in the IRP may, in the future, be applied to treatment of alcohol-induced brain damage and development of pharmacologic agents that may control the level of alcohol ingestion.

Another area among the research endeavors of the IRP which has implications, not only for the understanding of alcohol-induced organ damage, but for general understanding of the factors that cause cell death, are the studies on calcium homeostasis being performed in the Laboratory of Metabolism and Molecular Biology. Our scientists have demonstrated that acetate, generated in large quantities during the metabolism of ethanol, can increase calcium accumulation in a number of organs, particularly within the cells of the liver. This calcium is stored, primarily, in mitochondria, and may accumulate in quantities that interfere with normal mitochondrial function. These are just a few of the



many outstanding studies being performed in the Intramural Research Program, and further detail of these, and other, studies can be obtained in the body of this annual report.

Even though the IRP has been laudably productive in its research endeavors during the last year, many of the scientists of our Program have functioned in severely constrained space and in space that is far from optimal for the sophisticated technology being applied within our research program. Space allocation to the NIAAA-IRP is in two widely separated locales. One of these locales, located in Rockville, has proved to be extremely problematic for accommodating the best of scientific endeavors. On the other hand, space constraints on the NIH campus have currently provided no alternatives for relocating our scientists in additional, contiguous space on the NIH campus. There does, however, seem to be some hope that future construction of additional space in the form of a multi-institute neuroscience facility on the NIH campus may allow for, relocating and integrating our whole IRP within the NIH.

The quality of the IRP research efforts continue to be well monitored by frequent reviews of the program by the Board of Scientific Counselors. During this last year, the Laboratory of Clinical Studies underwent an extensive review. The Board of Scientific Counselor's reports have been most helpful to the Scientific Director for guiding the Intramural Research Program's efforts, and the current members of the Board, Drs. Harold Kalant, Richard A. Deitrich, Paul Greengard, William Lands, Alton Meister, Jack H. Mendelson, and Hyman J. Zimmerman, have to be commended for their diligent service to our program. Three members of the Board of Scientific Counselors completed their term of service during the last fiscal year, and three new members of the Board have been added. Drs. Deitrich, Greengard and Mendelson replaced Drs. Goldstein, Boll, and Lindsley.

The IRP has, in addition to its research endeavors, its publications, and its presentations at scientific meetings, committed itself to increasing the scientific tools for alcoholism and other research, and to activities in the area of knowledge transfer which would benefit other researchers and alcoholism treatment and prevention specialists. Dr. Norman Salem, Jr., of our Intramural program, was an integral member of a group that developed the Fish Oil Test Materials Program. This program is a collaborative effort between NIH, ADAMHA and the Department of Commerce to provide materials for research involving polyunsaturated fatty acids derived from fish oils. The metabolism of such fatty acids has been hypothesized to be affected by alcohol and the availability of Fish Oil Test Materials to alcohol researchers, will allow for further inquiry into alcohol's actions on cell membrane fatty acid constituents.

A major meeting was organized by the Clinical Director and Scientific Director of the IRP as part of the NIH Centennial Commemoration. Well over 500 treatment and alcoholism prevention

specialists attended the program, "Alcohol Research From Bench to Bedside," which featured presentations by the best intramural and extramural alcoholism researchers. The transfer of information between scientists and clinicians certainly aids progress in both areas of endeavor. Both the Scientific Director of the IRP and staff have, during the course of the year, presented research seminars to a number of interested alcoholism constituency groups. Among these were the Scientific Director's presentation to the Coalition of Addictive Diseases where he discussed "What Research Tells Us: Implication for Treatment and Prevention." The Scientific and Clinical Directors also co-organized the AMSAODD Conference on Alcohol Research and presented talks on "Mechanisms and Modulation of Alcohol Tolerance" and "Neurotransmitters, Alcohol and Violent Behavior." In addition, Dr. Jeannette Johnson organized the ARUS/RSA all day symposium on "Psychosocial Characteristics of Children of Alcoholics." It seems clear that the IRP's efforts during the past year have spanned the gamut from performing the best research to providing for education and training of alcoholism research and treatment/prevention specialists.

Annual Report of the Laboratory of Clinical Studies  
National Institute on Alcohol Abuse and Alcoholism  
October 1, 1986 to September 30, 1987  
Markku Linnoila, M.D., Ph.D., Chief

## Introduction

During fiscal year 1987, investigators in the Laboratory of Clinical Studies continued the longitudinal projects outlined in previous annual reports and started new projects in the areas of panic disorder, compulsive gambling, alcohol withdrawal and depression in alcohol dependent patients. The occupancy rate of the 10-bed research ward has continuously exceeded 70 per cent, which is close to a practical maximum.

The laboratory is also investigating inheritance, family system effects, central nervous system and liver effects, and other complications of alcohol abuse and dependence. It is also testing novel approaches to the treatment of alcohol dependence and its complications.

### 1. Section of Clinical Science

The main objectives of the research in this section are:

- a. Description of the early and prolonged components of the syndrome which follows acute withdrawal in alcohol dependent patients, and assessment of central and peripheral indices of sympathetic/stress axis responses as targets for the action of ethanol, or possible predisposing factors in alcoholism.
- b. Elucidation of physiological and psychological functions in patients with alcohol amnesic syndrome and with alcohol-induced dementia, and the treatment of such patients with a specific serotonin uptake blocker, fluvoxamine.
- c. Behavioral and biochemical description of panic attacks, anxiety, depression, and suicidal behavior in alcohol dependent patients, and similarities and differences between alcohol dependence and compulsive gambling.

The main findings are that patients with a tendency towards impulsive behaviors have indices of low central nervous system serotonin turnover (Virkkunen M, Nuutila A, Goodwin F, Linnoila M: Arch. Gen. Psychiatry, 44: 241-247, 1987). Alcohol dependent patients may also have abnormal benzodiazepine receptor sensitivity (studies in progress). Serotonin uptake inhibition produced by fluvoxamine seems to improve certain memory functions in patients with alcohol-induced amnesia (studies in progress).

The Outpatient Clinic screens prospective patients and family members and follows up patients in long-term treatment protocols. Alcohol dependence has a hereditary component, and the risk of becoming alcohol dependent is exacerbated by social stressors. Therefore, the Laboratory is examining relationships of the following independent variables to the length of abstinence in alcohol dependent patients:

- a. New pharmacological maintenance treatments, mainly neurotransmitter-specific antidepressant drugs and transmitter precursors, some of which alter consumption of alcohol in animals and the acute effects of alcohol in healthy humans;
- b. Specific group and psychotherapeutic interventions.

A clientele of about 80 alcoholics has been in follow-up during fiscal year 1987.

## 2. Unit of Family Studies

The family studies program evaluates subjects for physiological and biochemical studies conducted by other investigators in the laboratory. Research currently in progress includes correlational studies comparing different subgroups of alcoholics in order to elucidate risk factors for alcoholism and impulsive behavior such as suicide attempts, physical violence and drug abuse. A study comparing family functioning in middle class black alcoholic families and non-alcoholic families began this year. The program is also in the process phenotyping large pedigrees of alcoholic families in collaboration with the Unit of Genetic Studies.

## 3. Unit of Genetic Studies

There is a strong genetic contribution to the risk of becoming alcohol dependent. Thus, work in the area of genetics involves linkage and association studies in patients, particularly members of intensively studied families with multigenerational alcoholism. Male alcoholics are also tested for impulsivity. Cell lines are established, and DNA and protein polymorphisms are used as genetic markers. Inbred mouse strains showing differences in behavioral sensitivity towards ethanol are analyzed to assist in identifying gene loci (Goldman D, Pikus H: Biochem. Genetics, 24: 183-194, 1986) which participate in determining such sensitivities. Furthermore, the investigators are studying alcohol dehydrogenases using monoclonal antibodies, enzyme methods and novel protein and DNA probes to better understand their structure and function.

Because violent behavior is prevalent among intoxicated alcohol abusing men and is associated with structural abnormalities of the Y chromosome, genetic studies will specifically elucidate chromosome markers associated

with this behavior. Probes for genes located on the Y chromosome have been obtained from other laboratories and developed on site. These studies are expected to run for several years, but they are potentially of great importance if they succeed in identifying genetic factors that control violent behavior in alcohol-abusing drinkers.

#### 4. Section of Clinical Brain Research

Investigators in the area of clinical brain research conduct sophisticated electrophysiological, neuropsychological, and brain imaging studies on alcoholics, individuals at high risk of developing alcohol dependence, heavy and light social drinkers, and abstinent carefully matched controls (Eckardt M, Rawlings R, Martin P: Prog. Neuro-Psychopharmacol & Biol. Psychiatry, 10: 135-144, 1986). Variables related to man-machine interactions and relevant to driving and occupational safety while intoxicated are also being studied (Lister R, Eckardt M, Weingartner H: In Recent Developments in Alcoholism (eds) Galanter M, Plenum Publishing Corp. 1987; Rohrbaugh J, Stapleton J, Parasuraman R, Frowein H, Eckardt M, Linnoila M: In Alcohol & Alcoholism, Pergamon Journals Ltd., 1987; Stapleton J, Guthrie S, Linnoila M: J. Stud. Alcohol, 47: 426-432, 1986; Eckardt M, Stapleton J, Rio D, George D, Rawlings R, Weingartner H, Linnoila M: 15th Collegium Internationale Neuro-Psychopharmacologicum, 1986). Novel and potentially effective countermeasures to reduce the safety risks produced by alcohol in traffic and occupational tasks will be tested.

New strategies to reverse the cognitive deficits associated with acute alcohol intoxication and with chronic alcohol dependence are being explored. In addition animal studies are being conducted on behavioral effects of alcohol and actions of alcohol antagonists (Lister R: Psychopharmacology, 92: 78-83, 1987; Lister R: Psychopharmacology, 92: 180-185, 1987; Nutt D, Lister R: Brain Research, 413: 193-196, 1987).

#### 5. Section of Clinical Biochemistry and Pharmacology

Because the liver is one of the major targets of alcohol-induced organ damage, researchers in clinical biochemistry and pharmacology are studying liver function and effects of impaired liver function on pharmacokinetics and pharmacodynamics of common medications in alcohol dependent patients (Guthrie S, Lane E: Alcoholism: Clin. Exp. Research, 10: 686-690, 1986; Insel T, Lane E, Sheinin M, Linnoila M: Eur. J. Pharmacology, 136: 63-68, 1987). These studies are expected to provide rationales for individualized drug dosing to treat patients with varying degrees of alcohol-induced liver injury. The long-term goal is to develop a noninvasive liver function test based on measurements of labeled carbon dioxide in breath after administration of  $^{13}\text{C}$ -labeled drugs.

Pharmacokinetic principles and clearance concepts are being applied to studies of disposition of neurotransmitters and their metabolites in humans in vivo. The goal is to define new testable hypotheses about the biochemical bases of alcohol dependence and mental disorders and the mechanisms of action of drugs used in their treatment. These methods have for the first time been used to characterize the pharmacokinetics of norepinephrine in humans. The results of such analyses are helpful in interpreting the meaning of concentrations of norepinephrine and its metabolites in various body compartments.

## 6. Section of Analytical Chemistry

Research in the section of analytical chemistry concerns modification of biological functions of polyunsaturated lipids by alcohol. Both plasma membrane structure and fatty acid metabolism are being examined (Salem N, Kim H-Y, Yergey J: In Health Effects of Polyunsaturated Fatty Acids in Seafoods, Academic Press, Inc., 1986). Prostaglandins, leukotrienes, and other oxygenated fatty acid metabolites are measured in cerebrospinal fluid from alcoholics and healthy volunteers (Yergey J, Kim H-Y, Salem N: Anal. Chemistry, 1344-1348, 1986). Investigators are also studying red blood cell membranes in alcoholics to evaluate changes in the species and molecular arrangements of phospholipids comprising those membranes (Kim H-Y, Yergey J, Salem N: J. Chromatography, 394: 155-170, 1987; Kim H-Y, Salem N: Anal. Chemistry, 9-14, 1986; Kim H-Y, Salem N: Anal. Chemistry, 722-726, 1987). These aspects of lipid metabolism and phospholipid function are known to be particularly sensitive to perturbation by alcohol.

Further progress has been made in the structural characterization of a novel series of leukotriene-like compounds formed in the central nervous system (Salem N, Kim H-Y, Yergey J: In Health Effects of Polyunsaturated Fatty Acids in Seafoods, Academic Press, Inc., 1986; Yergey J, Kim H-Y, Salem N: Anal. Chemistry, 1344-1348, 1986). The synthesis of these hydroxylated eicosanoids is stimulated by low doses of ethanol. In testing the effects of ethanol in the cardiovascular system it was found that altered contractility of the heart, and blood pressure, after ethanol exposure may have a common etiology in the disruption of prostanoid metabolism (Karanian J, Salem N: Alcoholism: Clin. Exp. Research, 10: 171-176, 1986; Karanian J, D'Souza N, Salem N: Life Sciences, 39: 1245-1255, 1986). These studies may eventually explain the association between a low incidence of atherosclerotic disorders and moderate consumption of alcohol and the increased incidence of cardiovascular disorders and stroke in alcohol dependent patients.

## 7. Section of Neurochemistry

A program of research in neurochemistry is providing logistical and practical support for clinical receptor function and neuropeptide studies in alcohol dependent patients, individuals at high risk of developing alcohol dependence, and healthy controls. The Section of Neurochemistry is also

conducting animal experiments and in vitro cell culture studies designed to elucidate the effect of alcohol on cellular functions, from membrane receptors to rate the of genomic transcription (Dave J, Eiden L, Karanian J, Eskay R: Endocrinology, 118: 280-286, 1986; Dave J, Eiden L, Lozovsky D, Waschek J, Eskay R: Endocrinology, 120: 305310, 1987; Ishac E, Eskay R, Hirata F, Axelrod J, Kunos G: Endocrinology, 120: 1073-1078, 1987; Dave J, Eiden L, Lozovsky D, Waschek J, Eskay R: Annals NY Aca. Sciences, 327-330, 1986; Dave J, Eiden L, Lozovsky D, Waschek J, Eskay R: Annals NY Aca. Sciences, 577-580, 1986). These studies are expected to provide rationales for new pharmacological treatments of alcoholism.

The Laboratory of Clinical Studies Administrative structure to support the comprehensive clinical research program on the causes and complications of alcohol dependence outlined above has been strengthened in 1987. Information relevant for improving prevention and treatment of alcohol dependence and its complications is accumulating rapidly and particularly important are the new findings indicative of major differences in stress responsive physiological systems between alcohol dependent patients and controls. Furthermore, relatively specific serotonergic deficits may play a role in antisocial behavior associated with alcoholism and in alcohol-induced amnesic syndrome (Korsakoff's psychosis).





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00246-04 LCS
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Measurement of Norepinephrine and its Metabolites in Various Body Compartments</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	E. Lane	Senior Staff Fellow LCS, NIAAA
Others:	M. Linnoila	Chief LCS, NIAAA
	I. Parashos	Visiting Fellow LCS, NIAAA
	A. Roy	Visiting Associate LCS, NIAAA
COOPERATING UNITS (if any) Section of Clinical Pharmacology, LCS, NIMH (M. Rudorfer, W. Potter)		
LAB/BRANCH Laboratory of Clinical Studies		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>           Attempts to characterize neurotransmitter systems in man have focused on quantification of serotonin (5HT), norepinephrine (NE), dopamine (DA), and several several metabolites of these monoamines in various body fluids (urine, plasma, CSF). These neurotransmitter systems are suspected to be functioning abnormally in a variety of disorders (alcoholism, depression, schizophrenia, etc). In order to understand the meaning of measuring concentrations of neurotransmitters and their metabolites, it is necessary to understand their pharmacokinetics in different body compartments. a) Plasma and CSF concentrations, as well as 24 hour urinary measures of NE, DA and 5HT and their metabolites (VMA, MHPG, NM, 5HIAA, HVA) were collected in placebo treated, depressed subjects, alcoholics, pathological gamblers, and healthy volunteers. Samples were also collected in depressed subjects following treatment with antidepressants. Utilizing these data, a model was formulated which describes the disposition of MHPG. b) Rat liver perfusion using two NE metabolites, NM and MHPG, have been used to determine the fraction of a metabolite being transformed by each of several metabolic pathways. A better understanding of these relationships will help identify differences in formation, metabolism and elimination of these neurotransmitters in humans. This will offer insight into the functioning of these systems, and into the abnormalities of function that result in the previously observed abnormal concentrations of these neurotransmitters and their metabolites in certain disease states such as alcoholism and alcohol withdrawal.         </p> <p>           This project has been combined with Z01 AA 00255-03 LCS.         </p>		



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00238-05 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

CSF Neuropeptides and Prostaglandins in Alcohol Withdrawal and Brain Disease

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Linnoila Chief LCS, NIAAA

Others: J. Yergey Senior Staff Fellow LCS, NIAAA

## COOPERATING UNITS (if any)

Laboratory of Clinical Neurogenetics, NIMH (W. Berrettini); VA Medical Center, Washington, D.C. (J. Hawley)

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.0

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Severity of withdrawal symptoms from alcohol was quantified in alcoholics admitted to the Neurology Ward of the Washington, D.C. VA Hospital. Cerebrospinal fluid (CSF) samples were repeatedly obtained early during withdrawal and after all symptoms had subsided. Concentrations of the monoamine neurotransmitter norepinephrine and its major metabolite MHPG were measured at NIH. Significant positive correlations were observed between indices of elevated norepinephrine turnover and several signs of alcohol withdrawal. We are continuing this work trying to identify causes for the noradrenergic dysregulation during alcohol withdrawal. Thus, we are measuring peptides and prostaglandins, known to participate in the regulation of the functioning of noradrenergic synapses simultaneously with norepinephrine. We are correlating the concentrations of these neuromodulators to concentrations of norepinephrine and MHPG in the CSF and to the severity of withdrawal symptoms in our patients. We have completed this phase of the study in six patients and are aiming to increase our sample size to fifteen.

PROJECT DESCRIPTION:Investigators:

M. Linnoila	Chief	LCS, NIAAA
J. Yerger	Senior Staff Fellow	LCS, NIAAA
W. Berrettini	Senior Staff Fellow	LCNG, NIMH
J. Hawley	Neurologist	VA Medical Center

Objectives:

Many symptoms of alcohol withdrawal are indicative of sympathetic nervous system overactivity (excitability, tremor, sweating, etc.). The activity of the sympathetic nervous system is controlled by central nervous circuits, which use norepinephrine as transmitter. We are investigating central noradrenergic activity in alcoholics undergoing severe withdrawal, and correlating the degree of clinical symptoms with biochemical changes indicative of the amount of norepinephrine released from presynaptic nerve terminals. Furthermore, we are measuring peptide and prostaglandin neuromodulators known to participate in the regulation of the noradrenergic neurone systems.

Methods Employed:

Clinical symptoms of withdrawal are rated by Dr. James Hawley in the Neurology Department at the Washington, D.C. VA Hospital where the patients are treated and studied under a VA approved protocol. The instrument used to quantify severity of individual withdrawal symptoms, as well as the total score, is the extensively validated Gross rating scale. Dr. Hawley also performs two LPs on every subject, one early and the other one late during withdrawal. CSF norepinephrine and MHPG concentrations are quantified in the Laboratory of Clinical Studies, NIAAA with liquid chromatography using electrochemical detection. Prostaglandins are quantified with mass fragmentography. Neuropeptides are measured with radioimmunoassays by Dr. Berrettini.

Major Findings:

CSF norepinephrine and MHPG concentrations were significantly ( $p < .01$ ) higher in the early than in the late CSF samples obtained from alcoholics during withdrawal. The concentrations in the early but not in the late samples were significantly ( $p < .01$ ) higher than norepinephrine or MHPG concentrations in the CSF of an age and sex matched control group, which consisted of neurological patients. CSF norepinephrine and MHPG concentrations related highly with each other and with the severity of sweating, anxiety, tremor, heart rate and blood pressure in the withdrawing alcoholic patients.

Significance to Biomedical Research and the Program of the Institute:

These results provide the strongest available evidence of significant central noradrenergic overactivity during alcohol withdrawal in humans. Furthermore, the significant associations between the biochemical results and clinical symptoms are suggestive of the role noradrenergic overactivity plays as a possible cause of these symptoms.

Proposed Course:

We are collecting cerebrospinal fluid, urine, and blood samples in patients with alcohol-induced chronic brain disease and in a new group of patients undergoing withdrawal from alcohol to investigate possible monoaminergic deficits. Such deficits, if found, will provide rationale for treatment strategies. Furthermore, we are quantifying prostaglandins and neuropeptides known to be associated with the presynaptic release of norepinephrine. Such measurements are expected to elucidate mechanisms involved in the dysregulation of the noradrenergic systems during withdrawal from alcohol and in alcohol-induced chronic organic brain syndromes.

Publications:

None



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00256-03 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

HPLC Methods for the Measurement of Neurotransmitters

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Linnoila

Chief

LCS, NIAAA

## COOPERATING UNITS (If any)

Laboratory of Chemistry, NIDDK (K. Kirk, K. Jacobson, A. Gusowsky)

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

0.2

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have developed novel chemical derivatization procedures to render biogenic amines lipid soluble and/or electroactive. The derivatives have been extracted into organic solvents and quantified with electrochemical detection. These methods have been applied to quantify serotonin, normetanephrine and phenylethylamine in human and nonhuman primate cerebrospinal fluid.

PROJECT DESCRIPTION:Investigators:

M. Linnoila	Chief	LCS, NIAAA
K. Kirk	Research Chemist	LC, NIDDK
K. Jacobson	Staff Fellow	LC, NIDDK
A. Gusowsky	Staff Fellow	LC, NIDDK

Objectives:

To develop quantitative methods for the measurement of putative neurotransmitters and their metabolites in femtomolar concentrations.

Methods Employed:

Specific amine reagents with or without electroactive functional groups are used to derivatise monoamine neurotransmitters and their methylated metabolites. The derivatisation products are extracted into organic solvents, separated from other similar compounds with liquid chromatography and quantified with electrochemistry. The methods have been applied to measure serotonin, normetanephrine and phenylethylamine concentrations in cerebrospinal fluid.

Major Findings:

We can reliably and routinely quantify serotonin and normetanephrine in human lumbar cerebrospinal fluid. Studies on the effects of various pathological conditions and pharmacological manipulations on cerebrospinal fluid serotonin and normetanephrine concentrations are in progress.

Significance to Biomedical Research and the Program of the Institute:

Cerebrospinal fluid serotonin and normetanephrine concentrations have been difficult to quantify. The availability of new and relatively simple methods to quantify these variables will facilitate understanding physiological and pathophysiological significance of neurone systems using serotonin and norepinephrine as transmitters. Phenylethylamine is a "traceamine" which is present in the central nervous system. It has pharmacological effects similar to amphetamine. Due to a high incidence of coaddiction to stimulants and alcohol in certain patient populations it is particularly interesting to try to understand phenylethylamine functions in alcoholics.

Proposed Course:

We will apply the new assays to study cerebrospinal fluid biochemistry in violent offenders, alcoholics and patients with alcohol-induced organic brain syndromes.



Publications:

Gjerris, A., Sorensen, A., Rafaelsen, O., Werdelin, L., Alling, C., Linnoila, M: 5-HT and 5-HIAA in cerebrospinal fluid in depression. J. Affect. Disorders, 12: 13-22, 1987.

Gusowsky, F., Jacobson, K., Kirk, K., Marshall, T., Linnoila, M: A new HPLC procedure for the detection and quantification of -phenylethylamine. J. Chromatography, in press.

Marshall, T., Jacobson, K., Kirk, K., Linnoila, M: Liquid chromatographic assay for cerebrospinal fluid normetanephrine. Life Sciences, 40: 1513-1521, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00257-03 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Neuroendocrine Studies in Offspring of Familial Alcoholics

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Linnoila Chief LCS, NIAAA

Others: T. George Senior Staff Fellow LCS, NIAAA

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☒ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Responses of thyroid stimulating hormone (TSH) to thyrotropin releasing hormone (TRH) have been studied in the offspring of familial alcoholics and age, sex and past alcohol exposure matched control children. Sons but not daughters of familial alcoholics were found to have exacerbated TSH responses to TRH infusions.

PROJECT DESCRIPTION:Investigators:

M. Linnoila	Chief	LCS, NIAAA
T. George	Senior Staff Fellow	LCS, NIAAA

Objectives:

We wanted to investigate the persistently blunted TSH response to TRH infusions in long-term abstinent alcoholics, described by Larsen and Prange, as a possible genetic markers for vulnerability to alcoholism.

Methods Employed:

In the preliminary study, fifteen sons and fifteen daughters of familial alcoholics and 30 matched control children received i.v. infusions of TRH while at bed rest in our Outpatient Clinic. Triiodothyronine, thyroxine, and TSH concentrations were quantified. The sample has been expanded to 72 subjects at the present time. We are aiming at a sample of 120 subjects to provide a definitive finding. This sample size was deemed necessary by statistical power calculations.

Major Findings:

Sons, but not daughters, of familial alcoholics had markedly higher TSH responses to TRH than their matched controls. No differences were found in prolactin growth hormone and T<sub>3</sub> responses between the children at risk for becoming alcoholics and controls.

Significance to Biomedical Research and the Program of the Institute:

This is the first male-limited neuroendocrine abnormality described in children of familial alcoholics. If confirmed in further studies, an exacerbated TSH response to TRH infusions may become useful for identifying individuals at a high risk of becoming alcoholics. Furthermore, the finding may be indicative of a primary serotonergic deficit in the boys at high risk of becoming alcoholics.

Proposed:

We are entering the subjects into a longitudinal study and are enlarging our population sample.

Publications:

Moss, H.B., Guthrie, S., and Linnoila, M: Enhanced TSH response to TRH in boys at risk for development of alcoholism. Arch. Gen. Psychiatry, 43: 1137-1142, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00258-03 LCS

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Violent Behavior, Neurotransmitters, Glucose Metabolism and Alcohol Abuse

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Linnoila

Chief

LCS, NIAAA

COOPERATING UNITS (If any)

Department of Psychiatry, University Central Hospital, Helsinki, Finland  
(M. Virkkunen); IRP, NIMH (F.K. Goodwin, L.G. Brown)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.4

PROFESSIONAL:

0.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects

☒ (b) Human tissues

☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have investigated neurotransmitter metabolites and glucose metabolism in incarcerated violent offenders, arsonists and healthy volunteers. We have found that low cerebrospinal fluid 5-hydroxyindoleacetic acid (5HIAA) concentrations and hypoglycemia during oral glucose tolerance tests are associated with each other and impulsive violent acts and fire setting.

PROJECT DESCRIPTION:Investigators:

M. Linnoila	Chief	LCS, NIAAA
M. Virkkunen	Senior Lecturer	University of Helsinki
F.K. Goodwin	Director	IRP, NIMH
L.G. Brown	Staff Psychiatrist	BPB, NIMH

Objectives:

To investigate biological variables associated with impulsive and violent behaviors as well as alcohol abuse in humans.

Methods Employed:

Cerebrospinal fluid neurotransmitters and neurotransmitter metabolites have been quantified in samples obtained from violent offenders, arsonists and healthy volunteers. The subjects have been administered oral glucose tolerance tests and MMPIs. Careful forensic psychiatry examinations have been performed on the subjects.

Major Findings:

Violent offenders have low cerebrospinal fluid 5HIAA concentrations compared to healthy volunteers. Arsonists have both low cerebrospinal fluid 5HIAA and 3-methoxy-4-hydroxyphenyl glycol (MHPG; the main central metabolite of norepinephrine) concentrations compared to healthy volunteers. Eleven of twenty arsonists became hypoglycemic during an oral glucose tolerance test.

Significance to Biomedical Research and the Program of the Institute:

Alcohol abuse is associated with a large proportion of violent offenses and arsons. It has also been associated with a low cerebrospinal fluid 5HIAA concentration in the past. We have demonstrated clear associations between low cerebrospinal fluid 5HIAA concentration, alcohol abuse and violent behavior. Furthermore, we have found in arsonists associations between low cerebrospinal fluid 5HIAA and MHPG concentrations, abnormal glucose metabolism and alcohol abuse. These findings if replicated by others can form a rational basis for treatment interventions in these heretofore difficult to treat individuals.

Proposed Course:

We are planning to start to obtain lymphocytes in future subjects to relate the described findings to possible Y-chromosome abnormalities. We will enlarge our sample to blood relatives of the index individuals and appropriate controls to investigate the heredity of these conditions. We have invited a study on circadian rhythms in violent offenders because of their frequent complaint of insomnia.

Publications:

Linnoila, M., Virkkunen, M., Roy, A: Biochemical aspects of aggression in man: In Bunney Jr., W., Costa, E. and Potkin S. (Eds.): Clinical Neuropharmacology. Raven Press, New York, 1986, pp. 377-379.

Virkkunen, M., Nuutila, A., Goodwin, F., Linnoila, M: CSF monoamine metabolites in male arsonists. Arch. Gen. Psychiatry, 44: 241-247, 1987.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00269-02 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biological Factors in Abnormal Bereavement

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Roy Visiting Associate LCS, NIAAA

Others: M. Linnoila Chief LCS, NIAAA

## COOPERATING UNITS (if any)

Biological Psychiatry Branch, NIMH (W. Gallucci and P. Gold)

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The biology of bereavement, a risk period for increased drinking, was studied in a group of 19 recently bereaved subjects. Plasma cortisol and ACTH levels were measured in response to an infusion of corticotropin releasing hormone (CRH). Bereaved subjects, when compared with normal controls, had both raised basal plasma cortisol levels and blunted plasma ACTH responses to CRH. These results show that an adverse life event can cause dysregulation of the stress responsive hypothalamic-pituitary-adrenal (HPA) axis. Adverse life events are of interest as they precipitate depression and suicidal behavior among alcoholics.

PROJECT DESCRIPTION:Investigators:

A. Roy	Visiting Associate	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
W. Gallucci	Technician	LCS, NIMH
P. Gold	Section Chief	LCS, NIMH

Objectives:

Depressive episodes are frequent among alcoholics. Up to 18% of alcoholics die by committing suicide and up to 40% of alcoholics attempt suicide. Depression is the most frequent correlate of both suicide and attempted suicide among alcoholics. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis occurs in up to 60% of depressive episodes. Adverse life events have been shown to be significantly associated with both depression and suicide among alcoholics. Therefore, we were interested in investigating the possible effect of an adverse life event on the HPA axis.

Methods Employed:

The life event of recent bereavement was chosen for study because it is often accompanied by an increase in drinking behavior, because of its unequivocally adverse nature and because it is easily dated retrospectively. Nineteen recently bereaved subjects who responded to a newspaper article were assessed using a structured psychiatric interview. They completed various rating scales. They were then given the corticotropin releasing hormone (CRH) test and their plasma cortisol and ACTH responses were measured.

Major Findings:

The total group of recently bereaved subjects, when compared with 34 normal controls, showed both raised basal plasma cortisol levels and blunted plasma ACTH response to infusion of CRH. This is the same pattern of response found among depressed patients. Half of the recently bereaved subjects did, in fact, meet diagnostic criteria for a major depressive episode. Bereaved subjects who had received psychotropic medications in the past, compared with those who had not, had more blunted plasma ACTH responses to CRH.

Significance to Biomedical Research and the Program of the Institute:

These results are the strongest available evidence that an adverse life event (bereavement) can cause dysregulation of the stress responsive hypothalamic-pituitary-adrenal (HPA) axis in humans. Furthermore, the lack of significant difference between the blunted plasma ACTH response to CRH in the depressed bereaved subjects compared with depressed patients suggests that recent bereavement can precipitate a depressive syndrome with biologic correlates and that some bereaved subjects may need psychopharmacologic intervention.

Proposed Course:

We plan to examine drinking behavior and plasma endocrine responses to CRH infusion in a new group of subjects who have been recently bereaved.

Publication:

Roy, A., Gallucci, W., Linnoila, M., Haley, C: The life event of recent bereavement as a model for depression: results of the corticotropin releasing hormone stimulation test. Arch. Gen. Psychiatry, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00270-02 LCS

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Impulsivity and Pathologic Gambling

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. Roy	Visiting Associate	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA
	B. Adinoff	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL:

1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Biological factors involved in the lack of control associated with impulsive behavior have been investigated. We have performed lumbar punctures and the glucose tolerance test (GTT) in a consecutive series of 18 pathologic gamblers. Concentrations of 5-hydroxyindoleacetic acid (5-HIAA), the metabolite of the monoamine neurotransmitter serotonin, have been measured in the cerebrospinal fluid (CSF). Other CSF monoamine metabolites and peptides have also been measured. Plasma levels of insulin and glucagon during the GTT will be quantified. Personality variables have been measured using three personality questionnaires. We will compare the pathologic gamblers with normal controls and alcoholics and correlate the biological factors claimed to be involved in impulse dyscontrol with the personality variables.

PROJECT DESCRIPTION:Investigators:

A. Roy	Visiting Associate	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
B. Adinoff	Senior Staff Fellow	LCS, NIAAA

Objectives:

An important subgroup of alcoholics are males with an associated impulsive anti-social personality disorder. Studies of other groups who also manifest disordered impulse control have revealed evidence suggesting central serotonin deficiency and dysregulation of glucose metabolism. We thus decided to investigate central serotonin activity in a not previously studied group manifesting poor impulse control, pathologic gamblers (who not infrequently also have histories of alcohol abuse).

Methods Employed:

A consecutive series of 22 individuals meeting DSM-III criteria for pathologic gambling have been studied. Eighteen of these patients have had a lumbar puncture and concentrations of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) and other monoamine metabolites quantified using high pressure liquid chromatography with electrochemical detection. A five hour glucose tolerance test with measures of insulin and glucagon has also been performed. CSF peptides are also being measured using radioimmunoassays. Personality variables have been measured using the Eysenck and Foulds personality questionnaires. Life events have been measured by Paykel's method.

Major Findings:

Preliminary examination of the data suggests that, although the total group of gamblers do not have lower CSF 5-HIAA levels than controls, there may be a subgroup of pathologic gamblers who have low CSF 5-HIAA levels. Also, we have found that gamblers, compared with controls, have significantly higher neuroticism and hostility scores. Furthermore, gamblers with a major depressive episode have had significantly more antecedent life events than controls - events usually caused by the destructive effects of their gambling.

Significance to Biomedical Research and the Program of the Institute:

This is the first study of central neurotransmitter function in pathologic gamblers and when completed will provide further data about the presence or absence of biological factors in impulsivity and in clinical states associated with a loss of control in the presence of certain stimuli similar to alcoholism.

Proposed Course:

We will continue the study until we have a series of approximately 25 subjects. As well as examining biological factors in an impulse disorder syndrome we will

be able to search for correlations between various biologic and personality variables. We will also be able to examine the psychosocial and biologic correlates of depression and suicidal behavior among these patients. These data may also reflect on the primary causes of depression and suicidal behaviour among alcoholics.

Publications:

None





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 AA 00231-05 LCS
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Central and Peripheral Nervous System Function in Abstinent Alcoholics		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	B. Adinoff	Senior Staff Fellow LCS, NIAAA
Others:	M. Eckardt	Section Chief LCS, NIAAA
	M. Linnoila	Chief LCS, NIAAA
	D. Nutt	Visiting Scientist LCS, NIAAA
	D. Flowers	Medical Staff Fellow LCS, NIAAA
	B. Ravitz	Medical Staff Fellow LCS, NIAAA
COOPERATING UNITS (if any) Human Genetics Br., NICHD (A. Mukherjee); George Washington Univ. (H. Weingartner); Clin. Psychobiol. Br., NIMH (W. Mendelson, L. Tamarkin); Lab of Neurorad. & Comp. Tomo., NINCDS (R. Brooks); Nuclear Medicine, CC (S. Larson); Biol. Psychiat. Br., NIMH (P. Gold)		
LAB/BRANCH Laboratory of Clinical Studies		
SECTION Section of Clinical Science		
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	2.5	2.5
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  Behavioral deficits of alcoholics have been conceptualized in terms of two neuropathologically distinct syndromes: alcoholic dementia and Korsakoff's psychosis (alcohol amnesic disorder). Alcoholic dementia is characterized by diffuse cortical damage primarily related to the neurotoxicity of alcohol; Korsakoff's psychosis is associated with subcortical lesions due to nutritional (thiamine) deficiency. Severe memory impairment with relative sparing of other intellectual functions distinguishes Korsakoff's psychosis from alcoholic dementia (which may be clinically indistinguishable from the most common cause of dementia, Alzheimer's disease). We have recently found that sleep in Korsakoff patients is characterized by a reduced REM latency compared to normal volunteers, whereas Alzheimer patients have normal latencies. Furthermore, delta sleep is reduced in Alzheimer's disease, but is normal in Korsakoff patients. We have also demonstrated reduced daily excretion of the major urinary metabolite of melatonin, hydroxymelatonin, in patients with Korsakoff's psychosis. This finding is suggestive of impaired pineal function. Genetic differences in thiamine metabolism may predispose patients to develop Korsakoff's psychosis. Most patients with Korsakoff's psychosis whom we have studied have had a transketolase with reduced affinity for thiamine pyrophosphate. The majority of alcoholics with cognitive impairment demonstrate features characteristic of both syndromes. Pharmacologic modulation of neurotransmitter systems may be effective in treatment of the subcortical syndrome, whereas alcoholic dementia may require treatment strategies similar to those in Alzheimer's disease. This protocol is intended to utilize clinical, neuro-radiological, physiological, and neuropharmacological tests to differentiate these two pathologic entities, to follow a longitudinal course, and to relate variables in treatment protocols to outcome.		

PROJECT DESCRIPTION:Investigators:

B. Adinoff	Senior Staff Fellow	LCS, NIAAA
D. Nutt	Visiting Scientist	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
D. Rio	Physicist	LCS, NIAAA
J. Rohrbaugh	Research Psychologist	LCS, NIAAA
N. Salem	Section Chief	LCS, NIAAA
B. Ravitz	Medical Staff Fellow	LCS, NIAAA
D. Flowers	Medical Staff Fellow	LCS, NIAAA
R. Brooks	Research Scientist	LCS, NINCDS
P. Gold	Section Chief	BPB, NIMH
S. Larson	Chief	NM, CC
W. Mendelson	Research Psychiatrist	CPB, NIMH
A. Mukherjee	Section Chief	HCB, NICHD
L. Tamarkin	Research Biologist	CPB, NIMH
H. Weingartner	Professor	GWU

Objectives:

Chronic organic brain syndromes due to alcoholism constitute the second most common cause of dementia in adults (approximately 10%), ranking next to senile dementia of the Alzheimer's type (40-60%). Currently, a large proportion of dementing illness can be diagnosed with certainty only by examining the microscopic structure of the brain at autopsy. The crosssectional clinical picture of alcohol-related cognitive decline may be difficult to distinguish from that of the more prevalent primary degenerative dementia (Alzheimer's disease). Chronic alcohol abuse may lead to two clinically and neuropathologically distinguishable syndromes: alcoholic dementia and alcohol amnestic syndrome (also called Korsakoff's psychosis). These two organic brain syndromes may represent extremes of the spectrum of cognitive impairments related to chronic alcoholism. Alcoholic dementia is characterized by global intellectual decline, whereas the salient clinical feature of the alcohol amnestic syndrome is a severe and persistent memory deficit with relative sparing of other intellectual functions. The majority of alcoholic patients have aspects of both syndromes; presumably the midline subcortical lesions due to thiamine deficiency may explain the amnestic component, whereas the diffuse bilateral cortical damage resulting from alcohol neurotoxicity explains the global cognitive loss. It has been postulated that polymorphisms of thiaminerequiring enzymes may influence which clinical syndrome predominates. Most of the patients with Korsakoff's psychosis in whom we have studied fibroblast transketolase have had an elevated  $K_M$  for thiamine pyrophosphate in comparison with fibroblast derived from normal controls. We have found that patients with relatively "pure" amnestic characteristics have demonstrated episodic memory impairments that resemble those found in depression and Parkinson's disease and are distinguishable from the semantic or knowledge memory deficits found in Alzheimer's disease. Furthermore, we have demonstrated significant differences in the pattern of sleep EEG

abnormalities in Korsakoff's psychosis patients compared with those with Alzheimer's disease. The sleep of Korsakoff patients resembles that of patients with depression (increased arousals and shortened REM latency). We postulate that treatment strategies directed toward modifying activation and arousal by pharmacologic modulation of neurotransmitter systems may be effective in treatment of the alcoholic amnesic syndrome. This situation is analogous to the benefits derived from pharmacotherapy in depression and Parkinson's disease, whereas alcoholic dementia requires treatment approaches similar to those in Alzheimer's disease.

#### Methods Employed:

We will study two groups of controls (healthy nonalcoholics and alcoholics abstinent for at least six months) and four groups of patients (detoxified alcoholics who have been abstinent from alcohol for at least one week; alcoholics withdrawn from alcohol who have been abstinent for at least three weeks; alcohol amnesic patients; and alcoholic dementia patients), using the following clinical, physiological, and neurochemical tests: (1) skin biopsy for culture of fibroblasts and measurement of thiaminerequiring enzymes (e.g., transketolase), using an assay developed by Dr. Mukherjee's laboratory (2) neuropsychological evaluation of patients to determine whether they are predominantly amnesic or demented (in collaboration with Dr. Weingartner); (3) norepinephrine response to orthostasis; (4) dose-response to norepinephrine infusion; (5) norepinephrine and endocrine responses to insulin tolerance test; (6) catecholamine and neuropeptide metabolism in cerebrospinal fluid versus plasma and urine; (7) vasopressin response to hypertonic saline infusion, (8) thyrotropin-releasing hormone and gonadotropin-releasing hormone stimulation test; (9) dexamethasone suppression test; (10) corticotropinreleasing hormone test; (11) circadian rhythms of melatonin, body temperature, and activity (in collaboration with Dr. Tamarkin); (12) sleep EEG (in collaboration with Dr. Mendelson); (13) therapeutic trial of the serotonin uptake blocker fluvoxamine; and (14) positron emission tomography, computerized axial tomography, and nuclear magnetic resonance imaging (in collaboration with Drs. Brooks and Larson).

#### Major Findings:

A number of important findings have emerged. In the orthostatic challenge test, and the saline infusion (vasopressin test) we have found no differences between alcoholics and normal controls nor any important differences (other than age-related) between alcoholics with different ages and drinking histories. These findings suggest that the peripheral control of blood pressure response to standing is unaltered in sober alcoholics and that any possible hypothalamic damage due to drinking does not extend to the osmo receptors.

Significant positive findings have also emerged. First, in the norepinephrine infusion it was demonstrated that the young alcoholics (20-29 years) had a blunted initial hypertensive response to norepinephrine. We are presently measuring plasma levels to determine whether this is a pharmacodynamic or pharmacokinetic difference. Interestingly, the older alcoholics did not show a

similar subsensitivity, perhaps suggesting that the finding in young alcoholics may reflect a trait value. The insulin challenge test has revealed that, again, in young alcoholics there is a relative subsensitivity of peripheral norepinephrine/epinephrine release. Epinephrine levels rose later in alcoholics than in controls and there was a significant reduction in total output. The alcoholics showed virtually no norepinephrine response, whereas controls showed a quite significant effect. In contrast the cortisol and growth hormone responses to the insulin challenge were identical in the two groups, and this reveals that central processing of the hypoglycemic signal is the same in the two groups. When these results are taken together with those reported above, the suggestion is that there is a peripheral defect of sympathetic nervous function relating to adrenal activity in alcoholics. This could reflect damage due to chronic alcohol use or could represent a trait marker for the condition, since it's been suggested that adrenal function in children at risk for alcoholism may be abnormal. Further studies are needed to clarify this point. The therapeutic trial with the serotonin uptake inhibitor fluvoxamine in patients with organic alcohol induced brain syndromes revealed some fascinating findings. The most important one was that patients with alcohol amnesic disorder (Korsakoff's syndrome) showed a significant improvement in certain memory tasks whilst on the drug. In some cases this improvement was clinically relevant, e.g., they remembered phone numbers whereas they previously did not. Subsequent analysis of plasma fluvoxamine levels showed that there was a strong positive correlation between plasma level and improvement in memory. This strongly suggests that memory improvement directly relates to the increased availability of serotonin in the synaptic cleft, and this suggestion is strengthened by the observation that changes in central serotonin turnover produced by fluvoxamine were greatest in the patients that showed the best response to the drug. This present study will be repeated titrating every patient to an optimal plasma level of fluvoxamine, to verify it, and to see whether in the patients who had not previously responded increased plasma levels will produce a therapeutic response. It should be noted that the patients with alcohol induced dementia showed no significant responses to the drug consistent with the idea that they have a more severe organic logical brain disease. The positron emission tomographic study using eighteen deoxyglucose on organic brain syndrome patients have demonstrated some evidence of abnormal glucose metabolism in these patients. However, numbers at present are small and need to be increased before a definite conclusion can be reached. Alcoholics administered CRH at one week ( $n = 11$ ) of abstinence demonstrated a significantly attenuated ACTH response to CRH compared to their responses after three weeks of abstinence. Nine of these patients demonstrated a significantly blunted ACTH response at both one and three weeks compared to controls ( $n = 15$ ), whereas two alcoholics showed a markedly accentuated response. In alcoholics with more than three weeks abstinence ( $n = 20$ ), ACTH response was similar to that of controls. Alterations in baseline cortisol levels and cortisol response to CRH were noted in all alcoholics, including those with greater than six months abstinence. Cerebrospinal fluid levels of CRH and ACTH in alcoholics abstinent greater than three weeks ( $n = 36$ ) did not differ from those of controls ( $n = 13$ ).

#### Significance to Biomedical Research and the Program of the Institute:

Chronic organic brain syndromes due to alcoholism are responsible for approximately 10% of dementia in the adult population. The fact that only a small

population of alcoholics develop complication of alcoholism suggests the importance of predisposing factors. We will attempt to identify genetic factors that may be predictive of which individuals will develop the alcohol amnestic syndromes if they abuse alcohol. We plan to develop a clinical, physiological, and biomedical classification system of alcohol-associated chronic organic brain syndromes; this system will have diagnostic, prognostic, and therapeutic applications.

If the improvement previously reported with fluvoxamine can be replicated in a followup study then this will have major implications for understanding the etiology and treatment of alcohol amnestic syndrome. It of course raises the question whether other forms of amnestic disorder such as those seen in association with negative symptoms of schizophrenia might also be ameliorated by 5HT uptake blockers.

The findings of abnormal peripheral sympathetic responsiveness, especially that of the adrenal gland in young alcoholics may help us understand the damage produced by alcohol. However, they may also reflect a trait predisposition in alcoholics which could conceivably be a biological marker of alcoholism risk, and may throw some light on the etiology of alcoholism. Clearly, we need to study nonalcoholic adult children of alcoholics to determine whether similar abnormalities exist, and thus to establish or refute whether this represents an inherited contribution to the disease. Further findings suggest that in most recently abstinent alcoholics the total ACTH response to CRH stimulation is decreased, and that this response tends to "normalize" over time. Although changes in the correlation of baseline cortisol levels and HPA axis responses to CRH were evident even in alcoholics with greater than six months abstinence. This suggests that alterations in HPA axis functioning may persist for prolonged periods following the cessation of drinking. It is not clear from this study if these findings are due to chronic effects of ethanol or to effects of the subsequent withdrawal syndrome.

Although elevated baseline free cortisol levels were not observed in any of the alcoholic groups studied, cortisol response to CRH stimulation was proportionately greater in the one week and three week abstinent low ACTH responders compared to controls. This observation is compatible with the development of hyperplasia and hyperresponsiveness of the adrenal cortex, which is known to occur after even a few days of stimulation with exogenous ACTH or with the application of experimentally induced stress. As the group of alcoholics we tested at one and three weeks abstinence had experienced no more than mild withdrawal symptoms, were in relatively good health, and were not tested until one week following their last drink, the findings reported are most likely quite subtle compared to the changes one might observe in a poorly nourished alcoholic following delirium tremens. Our observations may, therefore, suggest that pituitary responsiveness may be severely impaired for a prolonged period of time following abstinence in more severely impaired alcoholics, and could contribute to the medical and psychological problems seen in this group.

Proposed course:

Approximately 60 DSTs, 100 spinal taps, 65 ambulatory EEGs, 85 saline infusions, 90 norepinephrine infusions, 90 lying/standing norepinephrines, 63 TRH/GNRH infusions, and 650 insulin infusions have been obtained. Present efforts are being directed towards obtaining further studies from age-matched controls and long-term abstinent alcoholics. In particular we are adding controls in the younger age groups to clarify the findings in the young alcoholics.

Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00260-03 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of Social Drinking on Blood Pressure

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Adinoff Senior Staff Fellow LCS, NIAAA

Others: R. Eskay Section Chief LCS, NIAAA  
J. Karanian Senior Staff Fellow LCS, NIAAA  
M. Linnoila Chief LCS, NIAAA  
N. Salem Section Chief LCS, NIAAA

## COOPERATING UNITS (if any)

Hypertension-Endocrine Branch, NHLBI (H. Keiser)

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Science

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

0.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hypertension is common in the adult population of the United States. It has been demonstrated to be associated with increased cardiovascular morbidity and mortality. Alcohol consumption is also prevalent and may play an important causative or contributory role in up to one-third of all hypertensives. The association between hypertension and alcohol consumption awaits causative explanation. Elucidation of the pathophysiology of the alcohol associated increment in blood pressure is the purpose of this study. Blood pressure will be measured using a 24-hour ambulatory monitoring system for several days in normotensive and hypertensive social drinkers during periods of usual alcohol consumption and abstinence. Subjects will be on a low monoamine diet for the duration of the study. Blood and urine samples will be obtained during baseline or usual alcohol consumption, abstinence, and return to baseline levels of alcohol intake for measurement of neurotransmitters, neuromodulators, and electrolytes involved in blood pressure regulation. Changes in these regulatory systems will be related to blood pressure in the three phases of the study.

PROJECT DESCRIPTION:Investigators:

B. Adinoff	Senior Staff Fellow	LCS, NIAAA
R. Eskay	Section Chief	LCS, NIAAA
J. Karanian	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
N. Salem	Section Chief	LCS, NIAAA
H. Keiser	Chief	HEB, NHLBI

Objectives:

The association between alcoholism and hypertension was first observed in 1915 among French Legionnaires. Over the last 15 years, many population studies have suggested that consumption of ethanol is associated with an increased prevalence of systolic and diastolic hypertension. This is most noticeable in those persons consuming three or more drinks per day. This relationship is independent of weight, tobacco use, stress, age, physical activity, and caffeine consumption. An ongoing controversy exists as to whether elevated blood pressure is a primary effect of ethanol or is due to the ethanol withdrawal syndrome. In favor of it being a primary effect is the linear relationship between alcohol intake and blood pressure, the fall in blood pressure with abstinence, and the return of hypertension with reintroduction of alcohol, all of which have been previously reported.

The mechanism of the relationship between alcohol consumption and blood pressure regulation is unknown. The elevated blood pressure in chronic alcoholics does not appear to be explained by abnormalities in the renin-angiotensin system, catecholamines, or cortisol. This study is designed to determine the effect of moderate ethanol intake and abstinence from ethanol on blood pressure monitored continuously in the subjects' natural environment and to elucidate the mechanisms underlying the blood pressure changes. Subjects will serve as their own controls during abstinence.

Methods Employed:

We will study normotensive and hypertensive men 21 years of age or older who have at least a one-month history of average daily alcohol intake of 45-90 grams, and do not meet DSM-III criteria for alcohol abuse. Subjects must have had a previous period of abstinence without serious withdrawal symptoms and be in good health with no significant abnormalities on clinical examination other than an elevated blood pressure. Subjects whose systolic and diastolic blood pressures are less than 145 mm Hg and 90 mm Hg, respectively, will be considered to be normal volunteers; subjects with blood pressures greater than this will be considered hypertensive.

All subjects will follow a low monoamine diet for the duration for the study. Subjects will be asked to continue their usual amount of alcohol consumption (45-90 grams of alcohol per day) while wearing ambulatory blood pressure and



activity monitors continuously for 48 hours. Three consecutive 24-hour urines will be collected for measurement of NA, K, Cl, Ca, creatinine clearance, cortisol, and catecholamine metabolite excretion rates. During the first two weeks of outpatient observation, subjects will monitor and record their own blood alcohol levels (BAL), using a portable hand-held breathalyzer, hourly after the first drink of the day until BAL returns to zero or the subject goes to sleep, and on awakening in the morning.

On the days of blood pressure monitoring, subjects will come to the Clinical Center at 8 AM and 4 PM. On arrival, a double stop-cocked i.v. line will be placed for blood sampling and BP will be monitored by a Dynamapp automatic blood pressure machine. After the subjects rest for two hours, blood will be withdrawn for homovanillic acid, norepinephrine, 3-methoxy-4-hydroxyphenylglycol, serotonin, epinephrine, vasopressin, adrenocorticotropin (ACTH), atrial natriuretic factor (ANF), prostacyclin, thromboxane, renin, angiotensin, magnesium, ionized calcium, and creatinine concentrations. Immediately after the blood sample is obtained, subjects will stand and blood pressure will be measured every minute for five minutes; at the fifth minute, blood will be drawn again for quantification of catecholamines, ANF, and vasopressin. Subjects will then be asked to refrain completely from alcohol use for two weeks. Blood pressure and activity will be monitored for the first 72 hours and the last 48 hours of those weeks, and urine and blood samples will be obtained as in the first part of the study. Following this abstinence period, subjects will once again return to their usual ethanol intake. Blood pressure and activity will be monitored continuously for the first 72 hours and then two weeks later for another 48 hours. Breathalyzer measurements will be made daily from the onset of drinking until the final blood pressure measurement is obtained two weeks later. Blood and urine will be studied when blood pressure is being monitored, as in the first part of the study.

#### Major Findings:

Ten normotensive individuals have been studied and the data are currently being analyzed. Two weeks of moderate ethanol intake does not cause hypertension in normotensive individuals. Resumption of drinking following a two week abstinence produces a significant fall in both diastolic and systolic BP with a rise in heart rate. A significant increase in blood pressure is seen after two weeks drinking as compared with the first three days. The mild pressor effect of ethanol is seen only in the morning when blood alcohol levels are zero. We are collecting the data on hypertensive social drinkers and completing additional studies on normotensive subjects.

#### Significance to Biomedical Research and the Program of the Institute:

In the Framingham study, 20% of the population had blood pressures greater than 160/95 mm Hg while 45% had values in excess of 140/90 mm Hg. The increased cardiovascular morbidity and mortality associated with hypertension has been well documented. Alcohol consumption is prevalent in the adult population: approximately 30% consume more than four drinks (approximately 60 grams) per week. Alcohol consumption may play an important causative or contributory role to elevated blood pressure in 5-30% of hypertensive persons. The causal mechan-

ism for the association between hypertension and alcohol consumption awaits an explanation. Information derived from this study will directly address this issue.

Proposed Course:

The project will continue for approximately two years.

Publications:

None

## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00261-03 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Pathophysiology of the Alcohol Withdrawal Syndrome

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Adinoff Senior Staff Fellow LCS, NIAAA

Others: M. Eckardt Section Chief LCS, NIAAA  
J. Rohrbaugh Research Psychologist LCS, NIAAA  
M. Linnoila Chief LCS, NIAAA  
E. Majchrowicz Research Chemist LPPS, NIAAA  
C. Marietta Physiologist LPPS, NIAAA  
F. Weight Section Chief LPPS, NIAAA

## COOPERATING UNITS (If any)

Walter Reed Army Institute of Research (T. Jerrells); Clinical Psychobiology Branch, NIMH (W. Mendelson, L. Tamarkin)

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Science

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

0.75

## OTHER:

0.25

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In this project, we explore the pathophysiology of two important clinical problems frequently observed in the chronic alcoholic during withdrawal: (1) disturbed sleep-wake cycle, and (2) increased susceptibility to infection. Twenty-four hour measurements of body temperature, motor activity, ambulatory ECG, and urinary catecholamine excretion will be performed on acutely withdrawing alcoholics during withdrawal. Although originally this study was designed to obtain these measurements during the first 72 hours of withdrawal, recent research from our ward indicated that this time frame should be altered. We are, therefore, evaluating biologic rhythms in 24 hours on days one, three, and eight of withdrawal. Also, plasma cortisol will be obtained every half-hour and plasma ACTH and melatonin will be determined every hour (rather than every three hours as originally proposed) during these periods. Because of limitation on the total volume of blood samples, lying-standing norepinephrine determinations will not be obtained. Alcoholic patients and age- and sex-matched controls will be evaluated for general immunocompetence to determine the effects of long-term alcohol abuse and withdrawal from alcohol on the immune system. Patients will be evaluated at regular intervals to investigate effects of varying durations of abstinence from alcohol on selected parameters of immunocompetence.

PROJECT DESCRIPTION:Investigators:

B. Adinoff	Senior Staff Fellow	LCS, NIAAA
G. Bone	Guest Researcher	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
J. Rohrbaugh	Research Psychologist	LCS, NIAAA
B. Ravitz	Medical Staff Fellow	LCS, NIAAA
D. Flowers	Medical Staff Fellow	LCS, NIAAA
F. Weight	Section Chief	LPPS, NIAAA
E. Majchrowicz	Research Chemist	LPPS, NIAAA
C. Marietta	Physiologist	LPPS, NIAAA
T. Jerrells	Immunologist	WRAIR
W. Mendelson	Research Psychiatrist	CPB, NIMH
L. Tamarkin	Research Biologist	CPB, NIMH

Objectives:

The alcohol withdrawal syndrome is characterized by pathophysiological changes in many organ systems. In this project, we explore the pathophysiology of two important clinical problems frequently observed in the actively withdrawing chronic alcoholic patient: (1) disturbed sleep-wake cycle, and (2) increased susceptibility to infections.

Methods Employed:

Upon entry into the study, patients who meet DSM-III criteria for alcohol dependence and give a positive breath sample for ethanol on admission will wear an activity monitor and a rectal temperature probe for continuous 24-hour monitoring. An indwelling venous catheter will be inserted in an antecubital vein for blood sampling every 30 minutes around the clock, for determination of melatonin, cortisol, and ACTH. Monitoring and sampling procedures will be conducted on the day of admission for 24 hours. All studies will be repeated for 24 hours on the third and eighth days following admission. Ambulatory EEGs will be worn on days two through three and seven through eight to monitor sleep EEG during withdrawal. Ambulatory EEG, body temperature, motor activity, and urinary catecholamine excretion will also be obtained following three weeks of abstinence.

In the present study, we also propose to evaluate general parameters of immunocompetence in alcoholic patients and age- and sex-matched controls. Lymphocytes from peripheral blood will be evaluated for their ability to respond to nonspecific mitogens, recall antigens, and allogeneic tissue antigens (mixed lymphocyte response). Alterations in the humoral immune response will be evaluated by measuring antibody production in culture after stimulation of cells with polyclonal activators such as pokeweed mitogen as well as measurement of lymphokine production, which is a function of the T-cells. Lymphokines of interest include interleukin 2 and gamma interferon, both of which can

be easily assayed and yield quantitative data. Other lymphocyte functions of interest include the generation of cytotoxic lymphocytes in the mixed lymphocyte response and the measurement of natural killer cells. Of particular importance will be the evaluation of relative proportions of T and B lymphocytes in the circulation and the helper and suppressor T-cell subsets, using well defined surface markers. These studies will be accomplished using fluorescent-activated cell sorter techniques and will address whether alterations in lymphocyte type induced by ethanol result in the immune alterations previously reported in alcoholics. Plasma obtained from lymphocyte isolation procedures will be saved and stored for evaluation of antibodies reactive against the patients' lymphocytes. Fluorescent-activated cell sorting techniques will be used to evaluate autoantibody reactivity.

#### Major Findings:

Twenty-four hour measurements of cortisol, ACTH, melatonin, urinary catecholamines, temperature and activity have been obtained on six patients. Cortisol and ACTH levels have been determined on four of these patients. In the three patients who demonstrated a moderate withdrawal syndrome, cortisol levels were markedly elevated on the first day of withdrawal compared to the seventh day (day one 50 - 100 percent above day seven). Two of these three patients demonstrated an absence of a circadian rhythm on the first day of withdrawal. Both the absolute levels and the circadian rhythm of cortisol returned to control levels by day seven. The fourth patient, who experienced only minimal withdrawal symptoms, demonstrated normal cortisol levels on each day of withdrawal. There were no consistent alterations in ACTH.

#### Significance to Biomedical Research and the Program of the Institute:

Sleep disturbance is an important clinical symptom of alcohol withdrawal. Decreased slow-wave sleep, frequent arousals, and abnormalities of rapid eye-movement (REM) sleep have been well documented during alcohol withdrawal; it has been suggested that early relapse in alcoholics following abstinence may be related to REM rebound. We have demonstrated that nocturnal synthesis and release of pineal melatonin is significantly inhibited during chronic ethanol administration in the rat. It has been postulated that melatonin synthesized in the pineal is a transducer from the hypothalamic circadian pacemaker which may regulate endogenous rhythms such as the sleep-wake cycle, body temperature, and other circadian neuroendocrine rhythms such as cortisol secretion. These preliminary findings of marked stimulation of HPA axis functioning during the ethanol withdrawal syndrome are similar to those observed during severe depression, malnutrition, Cushing's syndrome, or operative stress. Signs and symptoms of ethanol withdrawal such as fatigue, weakness, hypertension, mental confusion, and depression, may be partially related to the excessive glucocorticoid levels observed during withdrawal. As cortisol levels were noted to be elevated even in the presence of a positive blood alcohol level, persons with alcohol dependence may experience elevations in cortisol on a daily basis as the ethanol level decreases (at night), contributing to long-term alterations in blood pressure, memory, immune function, mood, and stress response. The absence

of a diurnal variation in cortisol levels on the first day of withdrawal may indicate dysfunction of the alcoholic's circadian pacemaker, suggesting discrete impairments in neuroendocrinologic functioning. Twenty-four hour levels of melatonin, temperature, and activity will help clarify these alterations. In humans, excessive ethanol consumption has been associated with a number of defects in specific and nonspecific immunologic mechanisms. Control of pyogenic bacterial infections is partially dependent on the nonspecific response of polymorphonuclear leukocytes; as found in animal studies, ethanol consumption has been shown to profoundly affect mobilization of these cells to sites of inflammation as well as the ability of these cells to phagocytize and kill bacteria. Other studies have shown that abnormalities in the cellular and humoral aspects of the immune system are present in alcoholics and volunteers after consumption of ethanol. These defects include an inability to respond to immunization with various antigens with a delayed-type hypersensitivity response and antibody production. This observed failure of the immune response might be due to the reported lymphopenia and depression of circulating T-cells associated with ethanol ingestion. Functional impairments in lymphocyte responses have also been reported using in vitro assays of mitogen-induced proliferation. Although the mechanisms of these impairments are unknown, it has been suggested that nutrition may play an important role. It has been reported that alcoholics show inappropriate autoimmune responses, as evidenced by the production of antibodies to small bowel epithelium and skin fibroblasts as well as a general increase in immunoglobulin levels. It has been further shown that immunization of alcoholics with a pneumococcal polysaccharide vaccine produces a significantly elevated antibody response. These data suggest that ethanol abuse might produce a selective defect in immunoregulation; in support of this hypothesis, it has been shown that alcoholic patients have an inability to generate suppressor cells in vitro. The possibility exists that one effect of ethanol on the immune system is an alteration of immunoregulatory networks leading to autoimmune responses including ethanol-induced liver disease.

#### Proposed Course:

This project is anticipated to continue and expand to study therapeutic interventions for the alcohol withdrawal syndrome.

#### Publications:

Jerrells, T.R., Marietta, C.A., Bone, G., Weight, F.F., and Eckardt, M.J.: Effects of ethanol on immune function. Clinical Immunology Newsletter 8: 142-143, 1987.

Jerrells, T.R., Marietta, C.A., Bone G., Weight, F.F., and Eckardt, M.J. Ethanol-associated immunosuppression. Advances in Biochemical Psychopharmacology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 AA 00263-02 LCS																												
PERIOD COVERED October 1, 1986 to September 30, 1987																														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Corticotrophin-Releasing Hormone Sites Binding on RBCS in Alcoholics																														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">B. Adinoff</td> <td style="width: 33%;">Senior Staff Fellow</td> <td style="width: 33%;">LCS, NIAAA</td> </tr> <tr> <td>Others:</td> <td>J. Dave</td> <td>Visiting Associate</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>R. Eskay</td> <td>Section Chief</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>L. Roehrich</td> <td>Psychologist</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>M. Linnoila</td> <td>Chief</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>D. Flowers</td> <td>Medical Staff Fellow</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>B. Ravitz</td> <td>Medical Staff Fellow</td> <td>LCS, NIAAA</td> </tr> </table>			PI:	B. Adinoff	Senior Staff Fellow	LCS, NIAAA	Others:	J. Dave	Visiting Associate	LCS, NIAAA		R. Eskay	Section Chief	LCS, NIAAA		L. Roehrich	Psychologist	LCS, NIAAA		M. Linnoila	Chief	LCS, NIAAA		D. Flowers	Medical Staff Fellow	LCS, NIAAA		B. Ravitz	Medical Staff Fellow	LCS, NIAAA
PI:	B. Adinoff	Senior Staff Fellow	LCS, NIAAA																											
Others:	J. Dave	Visiting Associate	LCS, NIAAA																											
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	M. Linnoila	Chief	LCS, NIAAA																											
	D. Flowers	Medical Staff Fellow	LCS, NIAAA																											
	B. Ravitz	Medical Staff Fellow	LCS, NIAAA																											
COOPERATING UNITS (if any)  None																														
LAB/BRANCH Laboratory of Clinical Studies																														
SECTION Section of Clinical Science																														
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892																														
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:																												
1.5	1.5																													
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             Alterations in the hypothalamic-pituitary-adrenal axis (HPAA) have been reported during the acute and chronic intake of ethanol as well as during the ethanol withdrawal syndrome. The integrity of the HPAA has typically been evaluated by the determination of plasma ACTH and cortisol following the administration of dexamethasone, subsequent to CRH stimulation, or diurnal rhythms. Specific binding sites for corticotrophin-releasing hormone (CRH) have recently been demonstrated in peripheral tissues and red blood cells (RBCs). Glucocorticoids have been demonstrated to alter CRH binding in peripheral tissues, and ethanol exposure causes a corresponding decrease in CRH binding sites on rat RBCs and anterior pituitary membranes. Therefore, it is thought that CRH binding to RBCs may provide an important clinical tool to indirectly assess CRH receptor levels in the pituitary gland. The determination of CRH binding sites on human RBCs from alcoholics (abstinent at least three weeks, N=17) and normal controls (N=17) at 8 AM and 8 PM have demonstrated increased CRH binding at 8 AM compared to 8 PM. Also, all alcoholics (with one exception) had significantly higher CRH binding in both the morning and evening that did normal controls. RBCs from the children of alcoholics and non-alcoholics have been obtained to determine if CRH binding sites are altered prior to the toxic effects of chronic alcohol use. CRH binding sites on RBCs and plasma cortisol and plasma cortisol and ACTH have also been evaluated every hour for twenty-four hours on normal controls and alcoholics during withdrawal. Preliminary data demonstrate a diurnal rhythm of RBC-CRH binding with a peak at 2 AM and a trough at 2 PM.           </p> <p>This study has been terminated.</p>																														





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00264-02 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sensitivity to Diazepam in Alcoholics and Children at Risk for Alcoholism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Adinoff Senior Staff Fellow LCS, NIAAA

Others:	M. Linnoila	Chief	LCS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA
	J. Johnson	Research Psychologist	LCS, NIAAA
	E. Lane	Section Chief	LCS, NIAAA
	R. Lister	Staff Fellow	LCS, NIAAA

## COOPERATING UNITS (if any)

Neuroscience Branch, NIMH (S. Paul); George Washington Univ., (H. Weingartner); National Eye Institute, IR (V. Matsuo)

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Science

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The benzodiazepine-GABA-chloride ionophore receptor complex has been demonstrated to be involved in the physiologic and psychologic effects of ethanol. Diazepam, a benzodiazepine, binds to this receptor complex, and demonstrates a cross-tolerance to ethanol. Recent studies have shown that diazepam-induced alterations in eye movements offer a useful measure of benzodiazepine receptor sensitivity in humans. Preliminary findings at the NIMH and NIAAA suggest an increased sensitivity to the effects of diazepam, as measured by saccadic eye movements, in alcoholics. This increased sensitivity appears to persist despite long-term abstinence (up to four years). This may suggest either long-term toxic effects of ethanol upon the benzodiazepine receptor or an alteration in the receptor that is present prior to the onset of alcohol abuse. In this study we will continue our studies of diazepam sensitivity in alcoholics as well as evaluating if this increased sensitivity to diazepam is present in persons "at-risk" for the development of alcoholism compared to persons without a family history of alcoholism. Subjects will also be evaluated for EEC, ERP (event-related potentials), body sway, vigilance, tracking, memory, mood assessment and expectancy, ACTH, cortisol, prolactin, and growth hormone response to diazepam.

PROJECT DESCRIPTION:Investigators:

B. Adinoff	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
J. Johnson	Research Psychologist	LCS, NIAAA
E. Lane	Senior Staff Fellow	LCS, NIAAA
R. Lister	Visiting Associate	LCS, NIAAA
D. Rio	Physicist	LCS, NIAAA
J. Rohrbaugh	Research Psychologist	LCS, NIAAA
J. Stapleton	Staff Fellow	LCS, NIAAA
S. Paul	Chief	NSB, NIMH
H. Weingartner	Professor	GWU
V. Matsuo	Staff Fellow	IR, NEI

Objectives:

To evaluate (1) benzodiazepine receptor sensitivity in persons with a history of alcohol dependence compared to normal controls, and (2) benzodiazepine receptor sensitivity in young males with an alcoholic biologic father (and non-alcoholic biologic mother) compared with young males who have no history of alcohol abuse in their family.

Methods Employed:

Alcoholics and age-matched normal controls were screened to rule out significant medical problems, psychiatric disorders, drug abuse, or history of sensitivity to benzodiazepines. Prior to being entered into the study subjects were evaluated to ascertain that saccadic eye movements were normal at steady state. On the day of the study, an intravenous catheter was placed in the forearm and diazepam was infused in doses of 25 ug/kg, 50 ug/kg, and 100 ug/kg. Each dose was infused over one minute, and doses were administered every 15 minutes. Prior to the initial dose and following each dose, subjects were evaluated for eye movement, prolactin, growth hormone, and mood. Young males (14-21 y/o) (1) with alcoholic biologic fathers and biologic mothers without a history of alcohol abuse, and (2) without a significant family history of alcohol abuse (no parents, siblings, grandparents or no more than one second degree relative with alcohol abuse) will be evaluated to rule out significant medical problems, psychiatric disorders, drug abuse, or history of sensitivity to benzodiazepines. Following a complete family history, developmental history, determination of impulsivity, and trait anxiety, subjects will partake in two infusion studies. Diazepam or placebo will be administered in random order at least two weeks apart. Doses of diazepam will be as described above, and subjects will be evaluated prior to infusion onset, after each dose, and at one and six hours after the final dose. Subjects will be evaluated for saccadic eye movements, EEG, ERP (event related potentials), body sway, vigilance, tracking, memory, mood assessment and expectancy.

Major Findings:

None

Significance to Biomedical Research and the Program of the Institute:

Ethanol and benzodiazepines (BZs) have a number of similar neuropharmacological and behavioral characteristics. Both have anxiolytic, muscle relaxant, anti-convulsant, and sedative/hypnotic properties and both exhibit cross-tolerance to one another's pharmacological effects. Clinically, the BZs are the most commonly used drug class for the treatment of the ethanol withdrawal syndrome. Therefore, it has been postulated that ethanol's effect on the BZ-GABA-chloride receptor-ionophore supramolecular complex may play a role in ethanol intoxication, tolerance, and dependence. The characterization of alterations in the sensitivity of benzodiazepine receptors in alcoholics may, therefore, help in our understanding of both the effects of chronic alcohol abuse and the etiology of alcoholism. The genetic basis for developing alcoholism has been well documented, with a point prevalence of 25% in male and 5-10% in female relatives of alcoholics compared to the general population estimates of 3-5% in men and 0.1% in women. One strategy that has been employed for identifying biological and psychophysiological characteristics that may predispose an individual to develop alcohol abuse is to determine in alcoholics those characteristics that are clinically abnormal and do not improve with abstinence, suggesting that these characteristics were abnormal prior to abusive alcohol consumption. Obviously, this strategy is based on the assumption that the consequences of alcoholism are somewhat reversible with abstinence, whereas pre-alcoholic abnormalities will not show much improvement. One example of this strategy involves the P300 component of the Event Related Potentials observed in certain cognitive paradigms. Alterations in the P300 component have been reported in detoxified adult alcoholics, and they do not return to normal with abstinence. This observation prompted Begleiter et al. to study P300 in 11 to 13 year old male children of alcoholics, resulting in the finding of reduced P300 amplitude. Our findings of increased sensitivity to diazepam in alcoholics, even following long-term abstinence, suggest that this sensitivity may pre-date the onset of alcohol abuse. The determination of altered benzodiazepine sensitivity in persons at risk for the development of alcohol abuse would therefore offer 1) the potential of specific psychosocial and/or pharmacologic interventions in a clearly identified population at risk for the development of alcohol abuse, prior to the onset of the illness, 2) a possible etiology in the predisposition to the development of alcohol abuse, and 3) a further understanding of the psychopharmacologic effects of alcohol.

Proposed Course:

Eight alcoholic and age-matched normal volunteers have been evaluated. No person's "at-risk" for alcoholism or members of the respective control group have been evaluated to date.

Publications:

None



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00265-02 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of Alprazolam, Diazepam, Clonidine, and Placebo upon Ethanol Withdrawal

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: B. Adinoff Senior Staff Fellow LCS, NIAAA

Others:	M. Linnoila	Chief	LCS, NIAAA
	E. Lane	Section Chief	LCS, NIAAA
	B. Ravitz	Medical Staff Fellow	LCS, NIAAA
	D. Flowers	Medical Staff Fellow	LCS, NIAAA
	D. Nutt	Visiting Scientist	LCS, NIAAA

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Science

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The ethanol withdrawal syndrome, which is partially characterized by an increased activity of the noradrenergic system, is at present most commonly treated with diazepam or chlordiazepoxide, both conventional benzodiazepines. Alprazolam, a new benzodiazepine, has been demonstrated to be successful in the pharmacotherapy of depression and anxiety disorders, in contrast to the conventional benzodiazepines. Alprazolam may have a particularly potent inhibitory action on the noradrenergic system. It can, therefore, be postulated that alprazolam may be an effective and specific treatment for the ethanol withdrawal syndrome. Clonidine, a conventional antihypertensive, has been used to successfully treat withdrawal from the opiates, and most recently, nicotine and alcohol. This study will compare the effects of alprazolam, clonidine, diazepam, and placebo on: 1) the signs and symptoms of the ethanol withdrawal syndrome, and 2) the noradrenergic overactivity of the ethanol withdrawal syndrome. Adrenergic activity will be evaluated by determinations of cerebrospinal fluid (CSF), and plasma, catecholamines and their metabolites, plasma norepinephrine laying and standing, and lymphocyte B-adrenergic receptor sites. Also, changes in CSF pH have been reported during ethanol withdrawal. We will, therefore, evaluate CSF pH both during acute withdrawal and following three weeks of abstinence.

PROJECT DESCRIPTION:Investigators:

B. Adinoff	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
E. Lane	Senior Staff Fellow	LCS, NIAAA
B. Ravitz	Medical Staff Fellow	LCS, NIAAA
D. Flowers	Medical Staff Fellow	LCS, NIAAA
D. Nutt	Visiting Scientist	LCS, NIAAA

Objectives:

To evaluate: 1) the clinical efficacy of diazepam, clonidine, and alprazolam compared to placebo in the treatment of the signs and symptoms of the ethanol withdrawal syndrome, 2) the adrenergic hyperactivity observed during ethanol withdrawal, and 3) the differential effects of diazepam, clonidine, and alprazolam upon the adrenergic overactivity during ethanol withdrawal. In addition, determinations of the changes in CSF pH during ethanol withdrawal will offer a better understanding of neurophysiologic alterations that occur during the ethanol withdrawal syndrome.

Methods Employed:

Upon admission to 3B-N, patients will be evaluated hourly for: 1) signs and symptoms of withdrawal with the CIWA test, 2) blood pressure and pulse, 3) breath alcohol level. An intravenous catheter with heparin lock will be inserted upon admission to the ward. CEC and electrolytes will be obtained immediately upon admission and the results will be evaluated prior to the onset of the study. When the patient demonstrates a significant withdrawal syndrome, the study will be initiated. Patients will be requested to remain supine for one hour. Following one hour of bedrest, 55 milliliters of blood will be withdrawn for catecholamines and lymphocyte B-adrenergic receptors. The subject will then stand for five minutes, during which time blood pressure and pulse will be automatically obtained once every minute. Following five minutes of standing, five milliliters of blood will be withdrawn for catecholamines. The patient will then be requested to lie down again for an additional hour. Twenty-three milliliters of blood will be obtained for catecholamines, neuropeptides, glucose, and electrolytes. A lumbar puncture will be performed. CSF will be analyzed for catecholamines, indolamines, neuropeptides, alkaloid condensation products, pH and prostaglandins. Following the lumbar puncture, the patient will be requested to remain at bedrest for six hours, and pharmacologic treatment for the alcohol withdrawal will begin. Patients will be randomly assigned to be administered either diazepam, alprazolam, clonidine, or placebo. Assignments will be made by pharmacy and patient assignment will be unknown to ward staff and physicians. Patients will be administered drug in a loading dose design. Medications will be administered q1 hr. until the signs and symptoms of alcohol withdrawal subside (CIWA < 10). The doses will be as follows: alprazolam 1 mg po q 1 hour; diazepam 10 mg po q one hour; clonidine .2 mg q 2 hours (the off hour will be a placebo). No more than 12 doses will be administered on an hourly basis. Drugs will be administered on subsequent days every 6 hours as needed if withdrawal symptoms recur. Blood pressure, pulse, and hydration status will be closely monitored.

If at any time during the study the subject appears to be in progressive, severe withdrawal (CIWA > 25, consistent blood pressure of systolic > 70 mmHg and/or diastolic > 110 mmHg) and does not respond to the assigned medication, the blind will be broken and the subject will be treated with the appropriate medications. CBC and electrolytes will again be obtained on the second day of hospitalization prior to continuation of the study.

On the second, third, fourth, and seventh day following the onset of alcohol withdrawal, blood samples for catecholamines, lymphocyte B-adrenergic receptors, and lying/standing norepinephrine will be obtained as above. All studies, including lumbar puncture, will be repeated in the third week of hospitalization. Five milliliters of blood will be obtained just prior to each dose (except the first) and every six hours for thirty-six hours after the last dose for plasma drug levels. Twenty-four hour urine collections, quantification of activity with activity monitors, and mood rating scales will also be obtained.

#### Major Findings:

Project is in its initial start-up phase.

#### Significance to Biomedical Research and the Program of the Institute:

The ethanol withdrawal syndrome is accompanied by signs of sympathetic nervous system overactivity, such as sweating, tachycardia, hypertension, and tremor. Increased levels of 3-methoxy-4-hydroxyphenylglycol (MHPG), the central metabolite of norepinephrine, have been reported in the cerebrospinal fluid (CSF) and blood of alcoholics during prolonged alcohol withdrawal. A significant positive correlation of MHPG concentrations in the CSF with heart rate, systolic and diastolic blood pressure, tremor, anorexia, and sweating (all common signs and symptoms of alcohol withdrawal) has been demonstrated in alcoholics during alcohol withdrawal. Levels of CSF norepinephrine are also increased during alcohol withdrawal, and the levels of norepinephrine and MHPG in the CSF decrease in parallel with the resolution of the signs of alcohol withdrawal. These studies strongly suggest that medications which decrease noradrenergic overactivity may be successful in ameliorating the signs and symptoms of alcohol withdrawal.

In North America, benzodiazepines are considered the treatment of choice for ethanol withdrawal. Of these, diazepam and chlordiazepoxide are still the most commonly used. Although diazepam and chlordiazepoxide are reasonably effective in controlling acute withdrawal states, their long half-lives and pharmacokinetics may result in persistence of toxic effects beyond the requirement for sedation. Patients requiring large amounts of diazepam or chlordiazepoxide to control severe withdrawal states often remain lethargic, ataxic, or confused for several days after the withdrawal state has resolved. Toxic effects may be accentuated in patients with severe hepatic dysfunction and in the elderly. Large doses of diazepam or chlordiazepoxide may cause respiratory decompensation in patients with obstructive pulmonary disease, a common complication in older alcoholics. Also, neither diazepam nor chlordiazepoxide have antidepressant effects beyond the reduction of depression-associated anxiety.

Alprazolam, a triazolobenzodiazepine, is a more potent anxiolytic and has less toxicity than diazepam. It has been suggested that alprazolam may have a more specific effect upon the noradrenergic system than diazepam. Alprazolam has also been reported to have antidepressant properties equivalent to imipramine, and to alter REM sleep parameters and CSF norepinephrine and serotonin in the same manner as classical antidepressants. Alprazolam, thus, comes close to the theoretical profile of an ideal drug for managing acute ethanol withdrawal states: (1) a possible specific inhibitory action upon the noradrenergic overactivity of ethanol withdrawal; (2) prompt onset of sedation following oral administration to achieve rapid intervention in the evolving withdrawal state; (3) relatively short half-life to permit rapid metabolism and excretion of the drug to minimize persistence of toxic effects; (4) little or no biological activity of intermediate metabolites; and (5) antidepressant effect to facilitate motivation for postwithdrawal rehabilitation efforts. These theoretical advantages have, however, not been tested in controlled clinical trials on treatment of alcohol withdrawal.

Clonidine, a specific  $\alpha_2$ -adrenergic agonist, is a commonly used antihypertensive. It is also frequently used for the treatment of opiate withdrawal. Recent studies indicate that clonidine may also be beneficial in the treatment of withdrawal from nicotine and alcohol. Because of its side effects profile, clonidine may offer a superior pharmacologic treatment in the management of acute ethanol withdrawal. The effect of clonidine on the adrenergic overactivity of ethanol withdrawal has not been evaluated. In addition to further elucidation of the adrenergic overactivity of the ethanol withdrawal syndrome, this study will help determine if alprazolam or clonidine is a safer, more specific, and more effective treatment for the ethanol withdrawal syndrome.

#### Proposed Course:

This project will continue for approximately two years.

#### Publications:

None



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 00249-04 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pharmacologic Reduction of Alcohol Consumption on Alcoholic Patients

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. George Senior Staff Fellow LCS, NIAAA

Others: D. Nutt Visiting Scientist LCS, NIAAA  
M. Eckardt Section Chief LCS, NIAAA  
R. Eskay Section Chief LCS, NIAAA  
B. Adinoff Senior Staff Fellow LCS, NIAAA  
E. Lane Senior Staff Fellow LCS, NIAAA  
M. Linnoila Chief LCS, NIAAA  
N. Salem Section Chief LCS, NIAAA

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Science

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Recent studies indicate that alcohol consumption is regulated by several interacting neurotransmitters, including the dopamine and serotonin systems. In a randomized double-blind design, chronic alcoholic outpatients will receive L-DOPA or L-5-hydroxytryptophan, both with the peripheral decarboxylase inhibitor carbidopa or placebo for a 1-year period. During this year, alcohol consumption, liver function, craving for alcohol, mental status, psychosocial functioning, and compliance with medication will be assessed at regular intervals. Prior to entry into the study, after 3 months, and at 1 year, the following procedures will be conducted to measure drug effects: (1) behavioral evaluation; (2) determination of concentrations of drugs, monoamines, hormones, and peptides in blood and cerebrospinal fluid; (3) orthostatic changes in heart rate, blood pressure, and plasma norepinephrine concentrations; and (4) assessment of plasma vasopressin response to saline infusion. Changes in alcohol consumption will be related to biochemical and behavioral parameters.

PROJECT DESCRIPTIONInvestigators:

D. George	Senior Staff Fellow	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
R. Eskay	Section Chief	LCS, NIAAA
B. Adinoff	Senior Staff Fellow	LCS, NIAAA
E. Lane	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
N. Salem	Section Chief	LCS, NIAAA

Objectives:

Over 90% of physicians in private practice prescribe drugs for the treatment of alcoholism, although valid studies demonstrating the efficacy of pharmacotherapy in this disorder are lacking. Successful treatment outcome may be defined as one or more of the following: (1) reduction of the amount of alcohol consumed; (2) retention of the patient in treatment; (3) improvement of social and family relations; (4) maintenance of employment and financial status; and (5) amelioration of the medical and psychiatric complications of excessive alcohol consumption. There is now considerable evidence that reduction in alcohol consumption is of fundamental importance and can be expected to decrease the frequency and severity of alcohol-induced organic disease and to favorably modify behavioral problems that lead to or result from excessive alcohol consumption. Since alcohol consumption is maintained by reinforcement, which has a neurochemical basis, it may be possible to modify drinking behavior by pharmacologic interventions that alter central neurotransmitter function. Recent studies indicate that both central dopaminergic and serotonergic mechanisms may influence ethanol consumption. Evidence for functional dopaminergic and/or serotonergic deficits in alcoholics suggest that therapeutic strategies using the dopamine precursor L-DOPA and/or the serotonin precursor 5-hydroxytryptophan may benefit some patients.

Method Employed:

Alcoholic outpatients will receive L-DOPA or 5-hydroxytryptophan both with the peripheral decarboxylase inhibitor carbidopa or placebo, for a 1-year period in a randomized double-blind parallel design. Compliance will be measured by pill count and determination of blood L-DOPA, L-5-hydroxytryptophan, and carbidopa levels. Alcohol consumption will be monitored by a drinking logbook compiled by the patient, interview questionnaires with the patient and a significant other, sequential serum gamma GT levels, breathalyzer readings at the time of clinic visits. A lumbar puncture for measurement of biogenic amines and peptides, orthostatic norepinephrine test, and plasma vasopressin response to saline infusion prior to and after 3 months' treatment and 1 year's treatment will allow neurochemical classification of patients and determination of whether patient and neurochemical characteristics are related to treatment outcome.

Major Findings:

To date, 36 patients have been admitted to protocol: 3 have successfully completed the 1-year study, 5 are actively participating and 28 have resumed drinking and dropped out.

Significance to Biomedical Research and the Program of the Institute:

To date, drugs to reduce alcohol consumption have not been adequately investigated, and existing studies suffer from several methodological difficulties. For example, no agent has been studied for longer than 3 to 6 months, an insufficient duration of time to determine whether the natural history of a chronic relapsing illness such as alcoholism is modified. It has been difficult to adequately measure compliance with medication and the major outcome variable of alcohol consumption. There has been no attempt to study the relationship between specific pharmacologic effects of drugs and treatment outcome. Finally, no studies have attempted to identify the neurochemical and patient characteristics which would permit a rational choice of the optimal drug for individual alcoholic patients. The design of the proposed study will allow us to address the majority of these difficulties and thereby provide information that will have direct applicability to alcoholic patients.

Proposed Course:

This project will be terminated with the addition of several more subjects.

Publications:

None



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00266-02 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Relationship of Psychopathology to Neurofunction in Alcoholics

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	D. George	Senior Staff Fellow	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA
	A. Roy	Visiting Associate	LCS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA
	D. Lamparski	Staff Fellow	LCS, NIAAA
	D. Goldman	Unit Chief	LCS, NIAAA
	B. Adinoff	Senior Staff Fellow	LCS, NIAAA

## COOPERATING UNITS (If any)

Clinical Psychobiology, NIMH (W. Potter); Biological Psychiatry, NIMH (T. Uhde)

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Science

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Alcoholism and affective disorders frequently occur in the same individuals and in members of the same family. This association may represent the co-existence of two common disease entities in a given individual due to chance or because (a) alcoholism results from self-medication of an underlying affective disorder or (b) depression results from toxic effects of alcohol abuse. A number of studies have attempted to unravel this complex interaction but many unanswered questions still remain. Commonly, the depression disappears within two weeks of abstinence, however, it may have a prolonged course similar to primary depressive illness. A causal relationship is inferred from studies which show an increased incidence of alcoholism in families of patients with affective disorder, a high incidence of affective disorder in the families of alcoholics and a high incidence of suicidal behavior associated with both affective disorder and alcoholism. Studies have shown that alcohol may acutely improve the sense of affective well-being but with continued intoxication this improvement may be reversed. During chronic experimental intoxication, alcoholics not only become increasingly depressed but also more anxious. Descriptive studies have shown a large percentage of withdrawing alcoholics experience generalized anxiety and phobic reactions. In this protocol we propose to characterize certain biochemical aspects of depression and anxiety as they occur in alcoholic patients. To do this, we will examine cerebrospinal fluid, plasma for norepinephrine (lying and standing), urine for catecholamine metabolites and employ pharmacological challenge paradigms using lactate, isoproterenol and chlorimipramine.

PROJECT DESCRIPTION:Investigators:

B. Adinoff	Senior Staff Fellow	LCS, NIAAA
D. George	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
A. Roy	Visiting Associate	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
D. Lamparski	Staff Fellow	LCS, NIAAA
D. Goldman	Section Chief	LCS, NIAAA
W. Potter	Chief	CPB, NIMH
T. Uhde	Senior Staff Fellow	BP NIMH

Objectives:

To elucidate relationships of alcoholism to other psychopathology by studying the phenomenology and neurochemical characteristics of depression and anxiety in alcoholics and their non-alcoholic family members.

Methods Employed:

Patients admitted to the study will meet DSM III criteria of the American Psychiatric Association for alcohol dependence and will have been abstinent from alcohol for at least two weeks. An examination by a psychiatrist and the Schedule for Affective Disorders and Schizophrenia (SADS) administered by a research social worker, will be used to make the diagnosis of past or present major affective disorder or anxiety disorder in alcoholic patients according to DSM III criteria. Age- and sex- matched control populations will consist of (1) normal volunteers, (2) abstinent alcoholic patients without a diagnosis of major affective disorder or anxiety and (3) adult offspring of alcoholics between the age of 18 and 75 without a diagnosis of alcoholism.

Cerebrospinal fluid will be examined for monoamines, their metabolites, and peptides. Pharmacological challenge paradigms employing isoproterenol and chlorimipramine will be used to explore respectively the adrenergic and serotonergic systems which have been implicated in depression and anxiety disorders. Lactate infusions, commonly employed to induce panic in susceptible individuals, will be used as a probe for anxiety. Chloride ion and placebo will be administered in a double blind design concurrently with lactate to observe the effects of chloride ion on anxiety.

Major Findings:

To date, 20 patients have completed the lactate infusion study (12 alcoholics with panic, 8 children of alcoholics with panic [COA]). Results showed alcoholics with panic are less likely to panic during a lactate infusion compared with children of alcoholics with panic. The panic response obtained in the COA group is consistent with that reported for panic patients in the literature. Lactate induced physiological changes (BP, pulse) as well as biochemical changes (pH, electrolytes lactate levels) showed no difference between the two groups thus yielding no obvious explanation to the reduced lactate response in

alcoholics with panic. Isoproterenol and chlorimipramine infusions were administered to 15 alcoholics, 12 alcoholics with panic, 8 depressed alcoholics and 8 controls. When isoproterenol was given to alcoholics with and without panic, the group with panic showed a reduced blood pressure and heart rate response suggests a reduced Beta receptor sensitivity. Biochemical measures (norepinephrine, c AMP and isoproterenol levels) and comparison between other patient groups await analysis. Chlorimipramine infusions were analyzed for changes in prolactin, cortisol and ACTH levels. Preliminary analyses showed a trend toward increased stimulation of cortisol in depressed alcoholics compared with alcoholics without depression in response to chlorimipramine. Other patient groups and biochemical measures await further analyses.

Ten alcoholics with and without panic plus 5 COA's had lumbar punctures. Analyses for catecholamine levels and metabolites, electrolytes, peptides, etc. are in process. Lying/standing determination for pulse, BP and norepinephrine levels show a trend toward reduced pulse response with standing and a concomitant increased production of norepinephrine in alcoholics with panic compared to alcoholics without panic.

#### Significance to Biomedical Research and the Program of the Institute:

Alcohol abuse may have an adverse affect on mood contributing to feelings of anxiety, depression and suicidal ideation. Frequently, in order to cope with these negative emotional states, the alcoholic increases his drinking which results in a physical and psychological compromised state. To date, there have been a few studies which have attempted to study the biochemical links between affective states and alcoholism. By understanding possible biochemical perturbations that arise from or contribute to alcoholism, we can gain a better understanding of the effects of alcohol and provide avenues for more effective pharmacological intervention.

#### Proposed Course:

Future plans are to increase the number in each patient group to 15 patients pending the analyses results of physiological, psychological and biochemical measures.

#### Publications:

None





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00271-01 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pharmacological Studies in Obese Rodents

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. Nutt Visiting Scientist LCS, NIAAA

Others: C. Gleiter Visiting Fellow LCS, NIAAA  
M. Linnoila Chief LCS, NIAAA

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Science

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The obese rodent provides an interesting experimental model for work in the area of alcoholism as well as obesity and diabetes in that some strains show increased alcohol preference that appears to be related to their degree of diabetes. We have begun studying several strains of obese mice in order to further characterize the pharmacological defects underlying these observations. In particular we have investigated the effect of electroconvulsive shock (ECS) on blood sugar levels since this treatment has been reported to improve glucose levels in diabetic humans. It was of interest to see whether ECS would also be an effective antidiabetic agent in this animal model prior to investigating its actions on alcohol intake. Furthermore since abnormalities of serotonin function have been reported in alcoholics as well as in mice made diabetic by destruction of pancreatic islet cells we plan to study the actions of serotonin active drugs (eg uptake blockers and agonists) on diabetic control, body weight and body temperature in these animals. These peripheral measures will be correlated with indices of central function.

PROJECT DESCRIPTIONInvestigators:

D. Nutt	Visiting Scientist	LCS, NIAAA
C. Gleiter	Visiting Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA

Objectives:

To investigate the pharmacology of the obese rodent in relationship to its alcohol preference, obesity and diabetes. In particular we plan to explore the mechanisms by which electroconvulsive shock (ECS) may ameliorate plasma glucose control in these animals. We shall use measures of body weight, body temperature and plasma glucose to follow the action of ECS on diabetic control. Subsequently we shall measure brain receptor binding and brain concentrations of several neurotransmitters thought to be of importance in glucose metabolism, namely serotonin and norepinephrine. We also plan to see whether drugs which act on these transmitters have similar effects to ECS on diabetic control. We shall administer, on a chronic basis, several serotonin and norepinephrine uptake blockers as well as pre and postsynaptic receptor agonists and antagonists to determine if they have effects on diabetic control similar to that of ECS. If they do then we shall follow up these observations by measuring central neurotransmitter function using receptor binding assays as well as by measuring brain concentrations of transmitters and metabolites. We also plan to study the effects of blood glucose levels and the above treatments on receptors that may control food intake using receptor binding of drugs such as the appetite suppressant mazindol.

Methods Employed:

The initial studies will be made in obese mice and if the results are interesting we shall extend them in obese rats. Blood glucose levels are assessed using a commercial glucometer which requires only a single drop of blood from the tail. Electroconvulsive shock is administered using ear clip electrodes and results in an immediate seizure with full amnesia. Brain receptor and neurochemical studies will be carried out using standard assay methods. If preliminary results suggest an important action of ECS on blood glucose then we shall implant indwelling cannulae into the jugular vein of obese rats to enable us to make daily measurements of plasma hormones such as insulin. The obese rodent provides an interesting experimental model for work in the area of alcoholism as well as obesity and diabetes in that some strains show increased alcohol preference that appears to be related to their degree of diabetes. We have begun studying several strains of obese mice in order to further characterize the pharmacological defects underlying these observations. In particular we have investigated the effect of electroconvulsive shock (ECS) on blood sugar levels in diabetic humans. It was of interest to see whether ECS would also be an effective antidiabetic agent in this animal model prior to investigating its actions on alcohol intake. Furthermore, since abnormalities of serotonin function have been reported in alcoholics as well in mice made diabetic by

destruction of pancreatic islet cells we plan to study the actions of serotonin active drugs (eg. uptake blockers and agonists) on diabetic control, body weight and body temperature in these animals. These peripheral measures will be correlated with indices of central function.

#### Major Findings:

At present we have verified that glucose levels in obese mice with diabetes resembles that of the human maturity onset type. Less effect was noted in those mice that have insulin deficient diabetes (similar to the human juvenile onset type). The improvement in blood glucose in the obese mice was not due to major changes in body weight or body temperature. It occurred after only a single seizure and persisted for several weeks. Blood glucose levels in the nondiabetic animals did not change significantly over the course of treatment.

#### Significance to Biomedical Research and the Program of the Institute:

These findings may help us understand the mechanisms of action of ECS and drugs which could be of value in treating diabetes. Furthermore, since the obese mice have a predilection to ethanol studies on them may elucidate the control of ethanol preference in mice. If we can determine the effects of our treatments on ethanol preference in these obese animals we may gain insights into the control of drinking behavior and possible means of manipulating it.

#### Proposed course:

So far we have studied about 50 obese animals. We plan to continue the ECS studies and begin exploring the actions of the serotonin uptake blockers in these mice. In addition we shall start the more detailed assessments of plasma hormone levels following ECS in the obese diabetic rat.

#### Publications:

None



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00233-05 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Family Studies of Alcoholism

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. Lamparski	Staff Fellow	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA
	V. Moore	Social Worker	LCS, NIAAA
	D. Garnett	Social Worker	LCS, NIAAA
	A. Roy	Visiting Associate	LCS, NIAAA
	D. Goldman	Unit Chief	LCS, NIAAA
	D. Nutt	Visiting Scientists	LCS, NIAAA
	D. Spiegler	Research Psychologist	DBE, NIAAA

## COOPERATING UNITS (if any)

Social Work Department, Clinical Center, NIH (D. Rooney); Division of Educational Research Programs, Behavioral Science Research Branch, NIMH (A. King)

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Science, Unit of Family Studies

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4

## PROFESSIONAL:

4

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Unit of Family Studies has two major functions (1) to recruit and assess alcoholics, controls and their families, for various investigators within the Laboratory of Clinical Studies; and (2) to conduct psychosocial studies of alcoholic families and their individual members. In the current year, Unit staff have focused on coding and entering onto a computer the data collected since the inception of the Laboratory. A series of correlational studies comparing different subtypes of alcoholics are underway. A preliminary study comparing suicidal versus non-suicidal alcoholics on clinical, psychosocial and family variables has been carried out. In addition, the Unit has begun a study examining middle class black alcoholic families. Unit staff have also been collaborating with the Unit on Genetic Studies in identifying and phenotyping several pedigrees for linkage analysis.

PROJECT DESCRIPTION:Investigators:

D. Lamparski	Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
V. Moore	Social Worker	LCS, NIAAA
D. Garnett	Social Worker	LCS, NIAAA
A. Roy	Visiting Associate	LCS, NIAAA
D. Goldman	Unit Chief	LCS, NIAAA
D. Spiegler	Research Psychologist	DBE, NIAAA
D. Nutt	Visiting Scientist	LCS, NIAAA

Objectives:

To accumulate and evaluate clinical and genetic data through comparative studies of alcoholic and normal control patients and their respective family members. To investigate the role of genetic and environmental influences through: (1) studies of multigenerational families, family process, and family systems, and their relation to the maintenance of abusive and addictive drinking behavior; (2) phenomenological studies of subgroups of alcoholics (women, blacks, and Hispanics); (3) comparisons between different alcoholic populations (i.e., those who attempt suicide vs. those who do not; those who become violent while drinking vs. those who do not); (4) longitudinal studies of children at risk, focusing on predictive factors and early detection; and (5) systematic studies of response to behavioral and psychotherapeutic intervention.

Methods Employed:

Assessment and data collection methodology includes use of the following: SADS-L, RDC-F, MMPI, Michigan Alcoholism Screening Test, Faces-III (Olsen) and Locke-Wallace Marital Adjustment Scale, as well as other instruments which will enable us to quantify biopsychosocial characteristics.

Major Findings:

A preliminary examination of 215 actively drinking and recovering alcoholics who sought participation in NIAAA was conducted. Alcoholics with a history of suicide attempts were compared to those with no such history or demographics, family history and drug and alcohol use. Alcoholics with a history of suicide attempts were younger, a greater proportion were females and from lower socio-economic levels. They also consumed a greater quantity of alcohol when drinking, exhibited an earlier onset of alcohol-related problems and used or abused other drugs more than alcoholics with no attempt history. Contrary to hypotheses, no differences in family history of alcoholism were found. Further analyses are planned comparing lifetime psychiatric history of the alcoholics and their relatives. Six families have been assessed in the study comparing middle class black families of actively drinking (N = 4) versus recovering male alcoholics (N = 2). The study examines individual functioning, family functioning and cultural values in intact families with at least one child available for study. Preliminary analyses show the actively drinking in greater psychological distress as measured by the SCL-90. No differences were observed between the wives of alcoholics. Additional families are now being recruited.

Significance to Biomedical Research and the Program of the Institute:

The long-term course will focus on better understanding of the interplay between biogenetic factors and environmental events, especially dysfunctional patterns of family interaction. The continued availability of graduate students and guest research investigators, and the training of staff in computer skills enhance the Unit's future research capabilities. It is anticipated that the analysis of our demographic and clinical interview data will determine additional research directions.

Publications:

Davenport, Y., and Adland, M.: Issues in the treatment of the married bipolar patient: denial and dependency. In Journal of the American Psychiatric Association, in press.

Lamparski, D., Roy, A., and Linnoila, M.: Correlates of suicidal behavior in alcoholics. Alcoholism: Clinical and Experimental Research, 11: 216, 1987.





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00234 05 LCS
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Genetic Studies of Alcoholism		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	D. Goldman	Unit Chief LCS, NIAAA
Others:	R. Cotton	Senior Staff Fellow LCS, NIAAA
	M. Linnoila	Chief LCS, NIAAA
	R. Lister	Visiting Associate LCS, NIAAA
	C. Rajagopal	Visiting Fellow LCS, NIAAA
	J. Stoll	NRC Fellow LCS, NIAAA
	D. Lamparski	Staff Fellow LCS, NIAAA
COOPERATING UNITS (if any) Biological Psychiatry Branch, NIMH (L. Goldin); Laboratory of Viral Carcinogenesis, NCI (S. O'Brien); VA Medical Center, Portland, OR (J. Crabbe)		
LAB/BRANCH Laboratory of Clinical Studies		
SECTION Unit of Genetic Studies		
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10	6	4
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input checked="" type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>             To identify unknown genetic loci determining alcoholism, we are testing for linkage or association between genetic markers and behavioral phenotypes. The probability of establishing linkage or association is being maximized by 1) focusing on human alcoholism with impulsivity/aggressivity as a prominent accompanying behavioral trait, 2) utilizing mouse genetic models and 3) using a larger panel of markers than has heretofore been available. Markers include DNA polymorphisms defined by probes specific for the Y-chromosome. In addition, we have mapped large panels of new human and mouse protein polymorphisms by two-dimensional electrophoresis. We have identified in humans more than 50 independent polymorphic loci expressed among the abundant proteins of lymphocytes, fibroblasts, erythrocytes and serum. A third of these variant human loci have been chromosomally mapped. A subpanel of 39 loci is being used for linkage analysis in families with alcoholism. In the mouse, we have identified 14 brain polypeptide variants and have preliminarily correlated one of these, which we mapped to chromosome 1, with ethanol intake (preference). We have chromosomally mapped four variant mouse brain polypeptides and identified twelve of the proteins visualized by two-dimensional electrophoresis of mouse brain. For correlative behavioral studies in collaboration with Dr. R. Lister (Clinical Brain Research), we have typed thirty strains of inbred mice at the 14 variant loci. We are studying the known enzymes of ethanol metabolism, principally alcohol (ADH). In this work, we have completed an RFLP linkage analysis between the three Class I ADH genes located on chromosome 4 and have demonstrated the existence of an ADH gene complex there using pulsed-field gradient DNA electrophoresis. We have shown that the only ADH present in brain is Class III ADH and have purified, characterized, and immunohistochemically mapped the human brain enzyme.           </p>		

PROJECT DESCRIPTION:Investigators:

D. Goldman	Unit Chief	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
R. Cotton	Senior Staff Fellow	LCS, NIAAA
C. Rajagopal	Visiting Fellow	LCS, NIAAA
J. Stoll	NRC Fellow	LCS, NIAAA
R. Lister	Visiting Associate	LCS, NIAAA
L. Goldin	Staff Fellow	BPB, NIMH
S. O'Brien	Chief	LVC, NCI
J. Crabbe	Staff Scientist	VA Med. Ctr., Portland, OR
D. Lamparski	Staff Fellow	LCS, NIAAA

Objectives:

Alcoholism arises from multiple genetic and environmental factors, the effects of which can usually only be observed in combination. Strategically, it is advantageous to study families because the environmental and genetic heterogeneities are less. In addition, inbred and outbred mice models can be used to study particular components in depth and molecular genetic methods offer the possibility of providing markers for the genetic determinants.

To maximize the extent of genetic homogeneity, we are studying large families also and individual males showing alcoholism associated with impulsivity/aggression. We have chosen this particular subtype of alcoholism (Type II) as the initial focus of study for several reasons: 1) studies done elsewhere indicate alcoholism associated with impulsivity/aggression is genetically transmitted, 2) the impulsive/aggressive phenotype may be, in both humans and animals, associated with lower central serotonergic activity and the finding of extra or abnormally long Y-chromosomes in males. This provides a possible connection to the observed greater prevalence of alcoholism in males (3-5%) than in females (0.1-1%).

The objectives of these studies are: 1) To use protein polymorphisms detected by two-dimensional protein electrophoresis and by other methods to establish molecular markers and locate genes responsible for alcoholism-associated behavioral differences. 2) To chromosomally map recently discovered polymorphic loci and to develop additional polymorphisms including ones at relevant loci such as tryptophan hydroxylase to improve the probability of establishing genetic linkage. 3) In humans and other animals, to study the molecular genetics and expression of relevant loci such as tryptophan hydroxylase and alcohol dehydrogenases. 4) To utilize protein polymorphisms and specific DNA probes for the Y-chromosome to study male alcoholism associated with impulsivity. 5) To isolate and study novel genetic differences relevant to alcohol consumption in humans and mice.

Methods employed:

Clinical studies of familial alcoholics and their relatives are being conducted in collaboration with the Unit on Family Studies (LCS, NIAAA), the Section of Clinical Brain Research (LCS, NIAAA) and Lynn Goldin (NIMH). Each family member receives a SADS diagnostic interview and structured family history and completes psychological tests including the MMPI (which yields subscale data normative for impulsivity), MFFT, and EPQ.

For protein and DNA genetic studies, a blood sample for establishment of a lymphoblast line is obtained. Sufficient cells are cultivated to provide material for analysis of protein polymorphisms by two-dimensional electrophoresis, and to obtain DNA for analysis with Y-chromosome and specific genic probes. Cells preserved in liquid nitrogen can later be grown for additional studies.

Polymorphisms are genetic variants with an allelic frequency of greater than 1% in the normal population. They can be used as markers in genetic linkage studies. Some variants, such as the known Oriental variants of alcohol and aldehyde dehydrogenases, are genetic determinants of human phenotypic differences. Protein polymorphisms by two-dimensional electrophoresis are detected by subjecting proteins of erythrocytes, serum, and cultured lymphoblasts to separation by isoelectric point in the first stage and separation on the basis of size in the second stage. Polypeptides are visualized by ultrasensitive silver stains or by autoradiography of isotopically-labelled proteins.

Polymorphisms of primary DNA sequence manifest themselves because they alter the sites where the restriction enzyme(s) cuts the DNA and therefore alter the sizes of the resulting DNA fragments. These restriction fragment length polymorphisms are detected by isolating DNA and digesting it to completion with the restriction enzyme of interest. The resulting DNA fragments are separated according to size by agarose gel electrophoresis. DNA in the gel is denatured and transferred to a nitrocellulose filter resulting in a replica of the pattern of fragments in the gel. To visualize the RFLP's, a cloned DNA sequence is radiolabeled and hybridized to the DNA on the filter, resulting in the detection of specific fragments. In this fashion, we have detected a single recombinant between the three Class I alcohol dehydrogenase loci residing on human chromosome 4. By employing the new method of pulsed-field gradient electrophoresis we have separated large fragments of DNA and shown that the 3 class I ADH genes reside on a single DNA fragment whose size was estimated.

The genetic linkage method depends on the demonstration of non-random genetic assortment of two genetically-determined traits. This occurs when the two traits are determined by chromosomal loci which lie close enough to each other so that they tend to not be separated by recombination. Dr. Lynn Goldin (NIMH) uses computer programs including LIPED to test for genetic linkage to localize disease and behavioral loci as well as newly described polymorphic molecular marker loci. For genetic linkage studies, the fraction of the genome which can be analyzed is largely determined by the number of polymorphic markers which are available. This is because a polymorphic marker locus must lie relatively close to a locus for this physical association to be proven by demonstrating nonrandom assortment in families. We are using a large (39) panel of protein

polymorphisms which are detected by two-dimensional electrophoresis of serum, erythrocytes, lymphocytes. This panel allows us to cover approximately 25% of the human genome at a linkage distance of 10 cM. We are also typing family members for DNA RFLPs.

The identity, characteristics, distribution and possible functional role of alcohol dehydrogenase in rodent and primate brain has been determined using immunohistochemical staining and enzyme purification and assay methods. Determination of enzyme parameters was accomplished by spectrophotometric assays with a variety of substrates and inhibitors. For immunohistochemical studies, specific polyclonal and monoclonal antibodies were raised against the individual Class I, II and III alcohol dehydrogenases purified in our laboratory. These antibodies were used for immunohistochemical staining of rat and primate brain sections and these results were confirmed using Western blotting. By a new method developed in our laboratory, we synthesized Capp Gapp, an affinity reagent for Class I alcohol dehydrogenase in order to demonstrate that this enzyme is totally absent from human and rodent brain and for rapid purification of Class I alcohol dehydrogenases.

For the mouse behavioral studies, we have identified fourteen polymorphic mouse brain protein variants by two dimensional electrophoresis. We have chromosomally mapped these loci by determining their phenotypes in recombinant inbred strains and have identified twelve other proteins on the mouse brain map. To identify variant loci determining alcoholism-associated genetic differences, we have used these brain protein variants to screen outbred mouse strains specifically selected for differences such as sleep time after ethanol administration (short sleep and long sleep) and severity of ethanol-withdrawal. These strains have been bred specifically to minimize inbreeding while the trait is selected so that any fixed genetic differences should relate to the trait for which the selection was conducted. In addition, replicate lines are studied when possible. Confirmatory studies involve determination of both the behavioral and the molecular phenotype in F2 generation hybrid animals. Additional mapping and behavioral correlation studies on inbred and outbred mouse lines are being done in collaboration with Dr. Richard Lister of the Section on Clinical Brain Research (LCS).

#### Major Findings:

Human protein polymorphisms by two-dimensional electrophoresis: in population and family studies, we had previously published on 40 protein polymorphisms by two-dimensional electrophoresis of human lymphocytes, fibroblasts, serum and erythrocytes. Approximately half of these were first identified by our group. We have now discovered additional lymphocyte and fibroblast protein polymorphisms so that the number detected is approximately 25 in each tissue, on a single gel. We have verified that most are transmitted in Mendelian codominant fashion and have shown that many of the polymorphic loci detectable in fibroblasts are also detectable in the lymphocyte.

Mouse models for alcoholism-associated behaviours: In the area of mouse genetic models for alcoholism-associated behaviors, we have published that fourteen genetic variant protein loci can be detected in mouse brain by two-dimensional

electrophoresis and that four of these loci show linkage to known markers so that their chromosomal position is known.

In work with Dr. J. Crabbe, VA Medical Center, Portland, OR and R. Lister (LCS) we have shown that one of 14 genetically variant mouse brain loci, an abundant 28 kilodalton, 5.6 isoelectric point protein mapping to chromosome 1, may be a determinant of ethanol preference (intake). In this work, we found the association by typing recombinant inbred mice produced from the C57BL/6J and DBA/2J cross mice and also in a group of 19 inbred strains chosen for their genetic diversity. We are attempting to confirm the linkage by analyzing a large number of F2 mice produced by the same cross.

Human familial alcoholism: Thirty-five adult male alcoholics have received psychological testing for impulsivity, and other traits, have had cell lines established in tissue culture and are being typed for protein and DNA polymorphisms. In these studies, Dr. R. Cotton is using restriction fragment length polymorphisms (RFLPs) specific for the Y-chromosome. Y-chromosome RFLPs are identified using 16 DNA probes, which recognize Y-chromosome-specific DNA fragments. These probes were isolated from a library of Y-chromosomal sequences obtained from the Lawrence Livermore National Laboratory.

Lymphoblastoid lines have been established from two large families with alcoholism collected in collaboration with the Unit on Family Studies. These families are being used for transmission and linkage analysis and additional families are being collected.

Human alcohol dehydrogenases: While in Unit of Genetic Studies, Dr. Rathnagiri purified to homogeneity Class III alcohol dehydrogenase from human brain, showed that Class III is the only alcohol dehydrogenase present in this tissue, and in collaboration with Dr. B. O'Neal (Laboratory of Neuropsychology, NIMH), immunohistochemically mapped the enzyme in primate brain. The mapping studies showed that the enzyme is not, as had been reported, found in particularly high concentrations in cerebellar Purkinje cells. Instead, it is highly concentrated in the subependymal layer in a region marked by fibers running parallel to the surface of the brain and is also widely distributed in lower concentrations. This finding was confirmed using the Western blot method and by enzyme assay. It would be of great interest to ascertain what function Class III alcohol dehydrogenase plays in intermediary metabolism. Of 24 alcohols, 24 aromatic and aliphatic aldehydes and three  $\omega$ -hydroxy fatty acids screened by Dr. Rathnagiri, the best substrates for the Class III enzyme were  $\omega$ -hydroxy and long chain fatty acids. By a new, rapid method, Dr. Rathnagiri has synthesized Capp-Gapp, an affinity reagent for purification of Class I alcohol dehydrogenases. He has used this reagent to confirm that Class I alcohol dehydrogenase is not present in human or rodent brain in even trace amounts. Cloning Class III ADH is in progress.

In a somewhat parallel effort (with S. O'Brien, NCI), we have developed a new method for determining genetic distances and phylogenetic relationships between species. In this method, several hundred proteins are compared between species for protein charge variation manifested on two-dimensional gels. In work recently published, we showed that the phylogenetic trichotomy of human, chimpanzee and gorilla should be broken in favor of the gorilla having diverged prior to the

separation of the other two lineages. We have provided a reconstruction of the phylogeny of all the living hominoid apes based on this powerful method and determined the rate of protein divergence over prolonged time scales.

#### Significance to Biomedical Research and the Program of the Institute:

Genetic studies in humans and in animals provide a paradigm for isolating biological factors which they can be studied with powerful molecular genetic methods. This type of approach can result in the discovery of genetic factors that participate in determining individual susceptibility to alcoholism. In the human, comprehensive clinical and psychological studies and establishment of cell lines from selected families provide the essential resource for linkage and transmission analyses.

The brain genetic studies in mouse strains differing in ethanol preference and response raise the possibility that a relevant behavioral locus (for preference) has been detected in the mouse which can then be searched for in human or primate studies.

Our identification of large numbers of protein polymorphisms by two dimensional protein electrophoresis and studies on Y-chosomal DNA polymorphisms contributes to the overall process of human gene mapping and characterization of human genetic variability. We are applying this resource of linkage markers to the mapping of genetic determinants of human alcoholism.

The purification and production of specific polyclonal and monoclonal antibodies to Class III alcohol dehydrogenase has provided tools for cloning of this enzyme, which is in progress.

The demonstration by Dr. Rathnagiri that Class III alcohol dehydrogenase is the only alcohol dehydrogenase present in brain definitively proves that ethanol is not metabolized by alcohol dehydrogenase to any significant degree in the brain. Dr. Rathnagiri's efforts to elucidate the role of Class III alcohol dehydrogenase in intermediary metabolism are of importance because of the possibility that ethanol may interact to disrupt the normal function of the enzyme.

Our phylogenetic work in which we have measured the molecular distances between the great apes in a new way, by two-dimensional electrophoresis, strengthens our general understanding of their phylogenetic relationships and of protein evolution. This work improves our ability to move extrapolate from non-human models to the human when genetic markers are identified.

Dr. Cotton's finding, using linkage and physical mapping approaches, that an ADH gene complex exists on chromosome 4, is a major step in understanding the expression, evolution, regulation and inheritance of these genes.

Proposed Course:

For human studies, collection of lymphoblast cell lines from intensively-studied families with alcoholism and from male alcoholics who have been psychologically tested and typed for impulsivity will continue. We are studying these families using the linkage method with protein and DNA molecular markers. We will continue to test individual male alcoholics with restriction fragment length polymorphisms specific for the Y-chromosome. Continued effort will be placed on identifying and chromosomally mapping new polymorphic loci discovered by the two-dimensional electrophoresis method for use as linkage markers.

We will continue the cloning work on Class III alcohol dehydrogenase.

In mouse brain genetic studies, we will attempt to confirm the correlation we have discovered between a genetic variant brain protein and ethanol preference. If it can be proven, we will search for variants at an analogous locus in the human. We will continue to expand our panels of genetic variant loci expressed in mouse brain and continue to seek linkages and correlations with behavioral loci of interest.

Publications:

Goldman, D., and Pikus, H.J.: Genetically variant, chromosomally mapped, and identified mouse brain proteins for behavioral analysis. Proc. of the IVth World Congress of Biol. Psychiat, in press.

Goldman, D., and Crabbe, J.: Use of Chromosomally mapped and identified mouse brain proteins for behavioral genetic analysis of alcoholism. Progress in Neuro-psychopharmacology and Biological Psychiatry 10:177-190, 1986.

Valkonen, K., and Goldman, D.: Preparative and analytical electrofocusing of class III human liver alcohol dehydrogenases in immobilized pH gradients. Protides of the Biological Fluids 34:785-789, 1986.

Goldman, D., and Pikus, H.J.: 14 Genetically variant proteins of mouse brain: discovery of two new variants and chromosomal mapping of four loci. Biochemical Genetics 24:183-194, 1986.

Goldman, D., and Linnoila, M.: Genetic approaches to alcoholism. Progress in Neuro-psychopharmacology and Biological Psychiatry 10:237-242, 1986.

Goldman, D.: Molecular markers for linkage of genetic loci contributing to alcoholism. Recent Developments in Alcoholism Vol. VI, in press.

Goldman, D., and Merrill, C.R.: Protein polymorphisms detected by two-dimensional electrophoresis: an analysis of overall informativeness of a panel of linkage markers. Journal of Psychiatry Research, in press.

Moss, H.B., Salin-Pascual, R.J., Giri, P.R., Goldman, D. and Tamarkin, L.: Sex-differences in ethanol sensitivity and alcohol and aldehyde dehydrogenase activities in the Syrian hamster, Alcoholism, in press.

Goldman, D., Rathnagiri, P., and O'Brien, S.J.: A molecular phylogeny of the hominoid primates as indicated by two-dimensional protein electrophoresis. Proceedings of the National Academy of Sciences, in press.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00239-04 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alcoholism-Associated Cognitive Impairment and Organic Brain Syndrome

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Eckardt

Section Chief

LCS, NIAAA

Others: R. Rawlings

Mathematical Statistician

DBE, NIAAA

## COOPERATING UNITS (if any)

United States Soldiers' and Airmen's Home, Washington, DC (N. Keller, A. Law, C. Smith)

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Brain Research

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS

1.5

## PROFESSIONAL

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this study is to examine longitudinally the neuropsychological performance of several clinically defined populations of detoxified male alcoholics. Comparisons will be made among detoxified alcoholics with clinically defined chronic organic brain syndromes, dementia or amnesic syndrome; less cognitively impaired alcoholics who are in an alcoholism treatment program; and nonalcoholic controls who are undergoing a routine physical examination. The risk of developing either type of chronic organic brain syndrome with continued alcohol abuse by alcoholics in a treatment program will be determined, as well as the consequences of long-term abstinence or continued alcohol consumption, albeit at significantly reduced levels.

PROJECT DESCRIPTION:Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
R. Rawlings	Mathematical Statistician	DBE, NIAAA
N. Keller	Staff Psychiatrist	MHS, USSH
A. Law	Chief	MHS, USSH
G. Smith	Chief	MHS, USSH
P. Martin	Assoc. Professor	Vanderbilt Medical School
H. Weingartner	Chairman, Psychology Dept.	George Washington University

Objectives:

Chronic alcohol abuse may lead to two clinically and neuropathologically distinguishable syndromes: alcoholic dementia and alcoholic amnestic syndrome (called Korsakoff's psychosis), which together constitute the second most common cause of dementia in adults (approximately 10%). These two alcohol-related organic brain syndromes may represent the extremes on a cognitive dysfunction scale with alcoholic dementia characterized by a global intellectual decline, whereas alcoholic amnestic syndrome can be characterized as a severe and persistent amnesia with a relative sparing of other intellectual functions. The majority of alcoholic patients in clinical practice fall somewhere in between. In the present study, we propose to use a comprehensive battery of neuropsychological tests to differentiate alcoholic dementia from alcoholic amnestic syndromes. Less cognitively impaired alcoholics will be evaluated similarly and then followed over time to determine whether (1) continued alcohol abuse results in a specific and predictable chronic organic brain syndrome, (2) abstinence results in improved cognitive functioning, or (3) continued alcohol consumption, albeit at reduced levels, results in an adverse effect on cognitive functioning.

Methods Employed:

Two clinically defined groups of alcoholics will be evaluated: Participants in an alcoholism treatment program and those with sufficient, clinically defined, cognitive impairment so as to be judged not likely to benefit from the treatment program. The latter group will be separated by neuropsychological performance into those with alcoholic dementia and those with alcoholic amnestic syndrome.

It has been shown previously that the neuropsychological performance of neurologically impaired alcoholics with dementia can be differentiated from that of alcoholics with amnestic syndrome. We plan to use a more comprehensive and sensitive test battery to better understand this differentiation. The neuropsychological scores for each alcoholic in the treatment program will then be compared to those with alcoholic dementia and those with alcoholic amnestic syndrome to determine to which group he is most similar. This designation will constitute the predicted outcome variable if that individual continues to abuse alcohol.

It is well established that abstinence can result in improved neuropsychological performance in mildly impaired alcoholics. Moreover, it has been suggested that continued alcohol consumption, albeit at significantly reduced levels, can reduce this rate of improvement. Some of the cognitive impairments in alcoholic dementia may also improve with abstinence, although the memory dysfunction in alcoholic amnesic syndrome is characterized as severe and persistent. The consequences of abstinence or reduced alcohol consumption will be determined for each of the three alcoholic groups by conducting annual neuropsychological testing. The nonalcoholics will also be subjected to repeated testing to control for any practice effects and for aging.

Initially, each subject will participate in four sessions on four separate days. The first three will involve a detailed neuropsychological assessment of intelligence, memory, and other cognitive functions. The fourth will involve the collection of socioeconomic information, personality assessment, childhood history of hyperactivity, and drug use history, including alcohol. At the end of the fourth session, each subject will be asked to provide the names of four collateral sources, friends or relatives, who can be contacted every six months to verify the patient's drinking and drinking-related behavior during the previous time period. Patients will be contacted monthly, interviewed in depth every six months, and subjected to annual neuropsychological testing for five years.

The neuropsychological test battery is designed to obtain a global assessment of cognitive skills, an in-depth examination of memory functions, and an assessment of alcoholism-related cognitive decrements. The examination will take about 12 hours to complete. The battery consists of Halstead-Reitan Battery including Trails A and B; Wechsler Adult Intelligence Scale; Wechsler Memory Scale; Wisconsin Card Sorting Test; and selected memory tests designed to compare episodic versus semantic learning, automatic versus effortful learning, and language versus nonlanguage learning.

Scales designed to evaluate MBD and hyperactivity during childhood will also be administered.

#### Major Findings:

We are collecting data and have not begun to analyze it.

#### Significance to Biomedical Research and the Program of the Institute:

It has been well documented that alcoholics have impaired brain function. The course of this impairment with continued alcohol consumption or abstinence has not been well studied and is one of the goals of the present research. Relatively little is also known about the etiology and reversibility of alcoholism-associated dementia which is the second most common form of dementia. Relationships between alcoholism-associated dementia and amnesic syndromes are unclear and are of importance in planning appropriate pharmacological intervention.

Proposed Course:

Data collection will be continued with anticipated preliminary results next year.

Publications:

Eckardt, M.J., and Martin, P.R.: Clinical assessment of cognition in alcoholism. Alcoholism: Clinical and Experimental Research 10:123-127, 1986.

Martin, P.R., Adinoff, B., Weingartner, H., Mukherjee, A.B., and Eckardt, M.J.: Alcoholic organic brain disease: Nosology and pathophysiological mechanisms. Progress in Neuropsychopharmacology and Biological Psychiatry 10:147-164, 1986.

Eckardt, M.J., and Martin, R.R.: Diagnosis and treatment of chronic organic brain syndromes associated with alcoholism. Substance Abuse (in press).

Martin, P.R., Mukherjee, A.B., and Eckardt, M.J.: Alcoholic organic brain disease. Proceedings of the IVth World Congress of Biological Psychiatry. New York, Elsevier (in press).

Linnoila, M., Eckardt, M.J., Durcan, M., Lister, R., Martin, P.R.: Interaction of serotonin with ethanol: chemical and animal studies. Psychopharm. Bulletin (in press).

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00240-08 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cognitive Function in Male Alcoholics

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Eckardt Section Chief LCS, NIAAA

Others: R. Rawlings Mathematical Statistician DBE, NIAAA

## COOPERATING UNITS (if any)

Department of Psychiatry and Human Behavior, University of California, Irvine  
(L. Gottschalk)

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Brain Research

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This series of studies is concerned with cognitive function in detoxified male alcoholics. Recent and chronic alcohol consumption variables were found to interact with each other and with age and education in a non-linear fashion in predicting neuropsychological performance. Increased consumption predicted decreased performance even on tests whose mean scores were in the normal range. Little or no improvement in performance was demonstrable with short-term abstinence (14 - 20 days), although long-term abstinence (7 months) was associated with improvement. Similarly, hepatic and hematologic characteristics of long-term abstainers improved, whereas these systems functioned abnormally in people who continued to consume alcoholic beverages, albeit at significantly reduced levels. Relationships between various pretreatment prediction variables and subsequent outcome are also being studied. Increased risk of relapse was associated with excessive drinkers who were relatively early in their alcoholic careers as assessed by years of abusive drinking and accumulated lifetime exposure to alcohol. Although statistically significant relationships were observed between scores on certain neuropsychological tests and posttreatment alcohol consumption, neuropsychological evaluation was determined to be of limited clinical utility.

PROJECT DESCRIPTION:Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
R. Rawlings	Mathematical Statistician	DBE, NIAAA
L. Gottschalk	Professor	Univ. of CA, Irvine

Objectives:

The present series of studies was designed to document the presence of CNS impairment in male alcoholics, discern possible etiological factors related to this impairment, and determine whether improvement in function is associated with subsequent abstinence. Additional questions concern whether treatment should commence immediately after detoxification, relationships between CNS function and treatment outcome, and neuropsychological consequences of post-treatment alcohol consumption.

Methods Employed:

A battery of 24 neuropsychological tests was administered to drug-free alcoholic inpatients (n=91) within 7 days of their last drink and again 17 days later. To control for practice effects, a nonalcoholic medical control group (n=20) also took the test battery twice, with approximately the same interval elapsing between administrations. Another group of alcoholic inpatients (n=32) took the tests only once, 14-31 days after their last drink. After patients completed the 21-day treatment program, they were contacted on a monthly basis to determine drinking behavior. At the end of 7 months, they returned to the hospital. Before testing, a breathalyzer and/or clinical laboratory determination of blood alcohol level was carried out in an attempt to ensure sobriety during testing. The 24 cognitive tests were then administered in a random order. Self-administered questionnaires were used to calculate post-treatment frequency of drinking alcohol and quantity consumed per occasion. Patient-supplied collaterals were then contacted to verify the patients' self-reports. Approximately 24 months after entrance into the treatment program, 17 of the original 91 patients were located and agreed to take again the entire battery of neuropsychological tests.

Major Findings:

Cognitive performance in drug-free alcoholic males is significantly predicted by chronic and recent drinking practices. Furthermore, it appears that certain patterns of consumption may accelerate the alcohol-induced decline of brain function. Little or no improvement in cognitive performance was demonstrable with short-term abstinence, when controls were included for the effects of repeated testing. Continued alcohol consumption by recovering alcoholics is associated with reduced cognitive performance, while those who abstained have improved test scores. Neuropsychological performances determined 24 months after entrance into the program were at the same levels as at 7 months after entrance.

Similar findings were observed in clinical laboratory tests, with long-term abstainers (7 months) having improved hepatic and hematologic functioning in contrast to the continued abnormal functioning observed in those people who continued to drink, albeit at significantly reduced levels. Further analysis of these clinical laboratory tests revealed widespread and persistent alcoholism-related alterations in organ system functioning even after long-term abstinence (7 or 24 months).

Male alcoholics' pretreatment levels of alcohol consumption were found to be related statistically to posttreatment levels of consumption with an increased risk of relapse associated with excessive drinkers who were relatively early in their alcoholic careers as assessed by years of abusive drinking and accumulated lifetime exposure to alcohol.

Statistically significant relationships were observed between neuropsychological test scores and posttreatment alcohol consumption determined eight months after completing treatment for 72 alcoholics. These results, however, were influenced by the particular measure of posttreatment consumption evaluated, type of statistical analysis, and whether the entire sample of 72 or a subsample of 55 with consistently reported drinking information was used. Scores of tests most consistently showing relationships were often counter to the notion that increased neuropsychological performance predicts more favorable treatment outcome. Discriminant analysis resulted in 70% correct classification, with chance being 50%. It is concluded that neuropsychological evaluation is of limited clinical utility in predicting posttreatment alcohol consumption.

#### Significance to Biomedical Research and the Program of the Institute:

Recent and chronic drinking practices appear to have adverse and possible direct effects on brain function in male alcoholics. Insofar as decisions about the initiation of therapeutic interventions which rely on cognitive processes are based on neuropsychological performance, we conclude that treatments may commence as soon as the clinical symptoms associated with acute withdrawal have subsided. Continued alcohol consumption by recovering alcoholics might serve to maintain cognitive performance at reduced levels, and this possibility should be considered in determining appropriate treatment goals for alcoholic patients. Neuropsychological evaluation is of limited clinical utility in predicting posttreatment alcohol consumption. However, it may be of value in assisting treatment staff in obtaining cognitively appropriate posttreatment employment for patients and in more effectively individualizing treatment, but this remains to be demonstrated.

#### Proposed Course:

Data analysis will be continued, and the results will be published in appropriate scientific journals.

Publications:

Eckardt, M.J., Rawlings, R.R., Graubard, B.I., Faden, V., Martin, P.R., and Gottschalk, L.A.: Neuropsychological performance and treatment outcome in male alcoholics. Alcoholism: Clin. Exp. Res. (in press).



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00267-02 LCS																												
PERIOD COVERED October 1, 1986 to September 30, 1987																														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Brain Imaging																														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI:</td> <td style="width: 40%;">M. Eckardt</td> <td style="width: 30%;">Section Chief</td> <td style="width: 10%;">LCS, NIAAA</td> </tr> <tr> <td>Others:</td> <td>J. Johnson</td> <td>Research Psychologist</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>M. Linnoila</td> <td>Chief</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>R. Rawlings</td> <td>Mathematical Statistician</td> <td>DBE, NIAAA</td> </tr> <tr> <td></td> <td>D. Rio</td> <td>Physicist</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>J. Rohrbaugh</td> <td>Research Psychologist</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>J. Stapleton</td> <td>Staff Fellow</td> <td>LCS, NIAAA</td> </tr> </table>			PI:	M. Eckardt	Section Chief	LCS, NIAAA	Others:	J. Johnson	Research Psychologist	LCS, NIAAA		M. Linnoila	Chief	LCS, NIAAA		R. Rawlings	Mathematical Statistician	DBE, NIAAA		D. Rio	Physicist	LCS, NIAAA		J. Rohrbaugh	Research Psychologist	LCS, NIAAA		J. Stapleton	Staff Fellow	LCS, NIAAA
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COOPERATING UNITS (if any) None																														
LAB/BRANCH Laboratory of Clinical Studies																														
SECTION Section of Clinical Brain Research																														
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892																														
TOTAL MAN-YEARS: <div style="text-align: center;">2.0</div>	PROFESSIONAL: <div style="text-align: center;">1.0</div>	OTHER: <div style="text-align: center;">3.0</div>																												
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <div style="border: 1px solid black; padding: 10px; min-height: 150px;"> <p>Various clinical imaging methods are being used to study the brain <u>in vivo</u>. These techniques enable comparisons of gross anatomy (CAT - Computed Axial Tomography; MRI - Magnetic Resonance Imaging) of the brain with electrical activity (EEG - electroencephalography; ERPs - Event-Related Potentials) and rate of glucose utilization in specific regions (PET - Positron Emission Tomography). From a clinical perspective, these techniques, in association with other diagnostic tests, enable qualitative judgments to be made as to the anatomic and physiologic integrity of the brain. In order to quantitatively analyze image data, the imaging techniques themselves are being investigated, as well as the effects of the associated mathematical models and subjective inputs on the reconstruction of the brain image. Moreover, mathematical and statistical methods for evaluating and relating these various sources of multivariate data are being developed.</p> </div>																														

PROJECT DESCRIPTION:Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
J. Johnson	Research Psychologist	LCS, NIAAA
E. Lamoreaux	Computer Programmer	ROB, NCI
M. Linnoila	Chief	LCS, NIAAA
R. Rawlings	Mathematical Statistician	DBE, NIAAA
D. Rio	Physicist	LCS, NIAAA
J. Rohrbaugh	Research Psychologist	LCS, NIAAA
J. Stapleton	Staff Fellow	LCS, NIAAA

Objectives:

Our goals are 1) to develop three-dimensional PET and CAT/MRI images which are superimposable, and 2) to develop new mathematical and statistical methods to assess these different types of multivariate image data and determine relationships between these images and brain electrical activity monitored at the scalp.

Methods Employed:

A critical review of the raw and reconstructed data obtained from PET, CAT and MRI is being carried out to insure that sources of noise inherent in each technique are taken into account thereby insuring that artifacts will be correctly eliminated and confidence intervals may be more accurately estimated, leaving only statistically significant differences. Emphasis is currently on self-attenuation within the brain-skull system, applicability of the glucose utilization model, and software filters used to reconstruct images.

Two of the main issues involved in this area are patient position monitoring during a scan and patient repositioning, either on the same scanner at a later time or on another scanner. Currently, two methods of recording patient position are being tested. The first system consists of a magnetic transducer attached to the patient, with associated pickup attached to the various scanners which would enable us to determine the position of a "stable point" on the patient and a computer system which will display this information both numerically and graphically. The second system consists of a mechanical system designed to ride on the patient's face to provide more quantitative visual cues as to patient movement without the complications of the aforementioned system.

All data obtained from the scanners are processed and displayed on our own image processing system enabling us to analyze basic pixel data instead of post-processed images. Individual brain slices are oriented in three dimensions and displayed as stacked three dimensional data or as surface contours. Using these methods it will be possible to display and calculate regional volumes and superimpose data from CAT, PET and MRI as well as other sources. This will insure that a regional alteration in metabolism corresponds to a particular anatomical location.

Resulting data are analyzed with mathematical techniques used in image processing, pattern recognition, and spectral analysis, i.e., by representing the spatial data in frequency space. Statistical tests are then used to study differences between various clinical populations.

Procedures are being developed to calculate and display scalp-monitored electrical potential fields and estimate the position and distribution of electrical sources in the brain producing these potentials. This will enable us to correlate anatomical or metabolic changes in the brain with modifications of cognitive processes as represented by changes in evoked responses.

#### Major Findings:

Data for this study are still being collected and analyzed.

#### Significance to Biomedical Research and the Program of the Institute:

Establishing structure and function relationships among various areas of the brain is a crucial step in determining mechanisms. The approach advocated in the research described herein emphasizes 1) detailed and intensive assessment of relatively few, carefully selected patients, thereby reducing heterogeneity in patient characteristics and enabling a convergence of information and 2) comparing three-dimensional PET and MRI images with each other and with electrical sources derived from scalp-monitored EEG and ERPs. Such studies have yet to be reported in the literature. Successfully combining these techniques would be a significant accomplishment with obvious applicability to other studies of brain structure and function.

#### Proposed Course:

Data are being collected and analyzed. As analyses are completed, the results will be published in appropriate scientific journals.

#### Publications:

None.



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00247-04 LCS
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Studies of the Offspring of Alcoholics		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	J. Johnson	Research Psychologist LCS, NIAAA
Others:	M. Eckardt	Section Chief LCS, NIAAA
	M. Linnoila	Chief LCS, NIAAA
	D. Rio	Physicist LCS, NIAAA
	J. Rohrbaugh	Research Psychologist LCS, NIAAA
	J. Stapleton	Staff Fellow LCS, NIAAA
COOPERATING UNITS (if any) Johns Hopkins University, School of Public Health, Dept. of Child and Maternal Health (J. Rolf).		
LAB/BRANCH Laboratory of Clinical Studies		
SECTION Section of Clinical Brain Research		
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.25	0.75
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Although alcoholism tends to run in families very little is known about how or why some family members express the risk towards alcoholism while other members of the family do not. The study of persons who are most at risk for alcohol abuse (that is, the children of alcoholics) can provide information helpful for understanding some of the behaviors which antedate the risk and protection from alcohol abuse. This study attempts to characterize the behavioral and CNS developmental patterns in the children of alcoholics by clinical, psychosocial and cognitive assessments. First degree relatives are also evaluated so as to compare patterns of functioning between the generations of related individuals sharing a common familial and genetic environment. The present research is designed to be a multivariate and developmentally relevant study of both the risk and protective factors associated with alcohol use and abuse among members of families in which one or both biological parents have been alcoholic.</p> <p>This project has been terminated.</p>		



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 AA 00268-02 LCS																					
PERIOD COVERED October 1, 1986 to September 30, 1987																							
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>The Behavioral Effects of Alcohol and Other Psychotropic Drugs</u>																							
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI: R. Lister</td> <td style="width: 40%;">Visiting Associate</td> <td style="width: 30%;">LCS, NIAAA</td> </tr> <tr> <td>Others: M. Durcan</td> <td>Visiting Fellow</td> <td>LCS, NIAAA</td> </tr> <tr> <td>M. Eckardt</td> <td>Section Chief</td> <td>LCS, NIAAA</td> </tr> <tr> <td>C. Gorenstein</td> <td>Guest Researcher</td> <td>LCS, NIAAA</td> </tr> <tr> <td>D. Goldman</td> <td>Unit Chief</td> <td>LCS, NIAAA</td> </tr> <tr> <td>M. Linnoila</td> <td>Chief</td> <td>LCS, NIAAA</td> </tr> <tr> <td>D. Nutt</td> <td>Visiting Scientist</td> <td>LCS, NIAAA</td> </tr> </table>			PI: R. Lister	Visiting Associate	LCS, NIAAA	Others: M. Durcan	Visiting Fellow	LCS, NIAAA	M. Eckardt	Section Chief	LCS, NIAAA	C. Gorenstein	Guest Researcher	LCS, NIAAA	D. Goldman	Unit Chief	LCS, NIAAA	M. Linnoila	Chief	LCS, NIAAA	D. Nutt	Visiting Scientist	LCS, NIAAA
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D. Nutt	Visiting Scientist	LCS, NIAAA																					
COOPERATING UNITS (if any) George Washington University (H. Weingartner); VA Research, Portland, OR (J. Crabbe); United States Soldiers' and Airmen's Home																							
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>To determine the psychobiological distinctiveness of various behavioral processes, pharmacological and genetic methods are being used. The relationships among alcohol's anxiolytic, reinforcing, amnestic, locomotor stimulant, sedative/hypnotic and hypothermic effects are being studied using a variety of inbred strains of mice. The role played by the benzodiazepine-GABA receptor complex in the effects of ethanol is also being investigated. Several benzodiazepine receptor inverse agonists have been found to partially reserve some of the behavioral effects of ethanol.</p> <p>A variety of different methods are being used to investigate mechanisms of learning and memory. The cognitive functioning both of normal human volunteers under the influence of different drugs (such as alcohol and benzodiazepines), and of various patient populations (e.g., Korsakoff's psychosis, various dementias) is being examined. The effects of drug treatments on learning and memory processes in laboratory rodents are also being studied. Finally the effect of changes in mood on cognitive function is being examined in normal volunteers following treatment with alcohol.</p>																							

PROJECT DESCRIPTION:Investigators:

R. Lister	Visiting Associate	LCS, NIAAA
M. Durcan	Visiting Fellow	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
C. Gorenstein	Guest Researcher	LCS, NIAAA
D. Goldman	Unit Chief	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
D. Nutt	Visiting Scientist	LCS, NIAAA
H. Weingartner	Professor	George Wash. Univ.
J. Crabbe	Staff Scientist	VA Med. Ctr., Portland, OR

Objectives:

The behavioral effects of alcohol in rodents have been most extensively investigated using tests sensitive to the drug's hypnotic, hypothermic, psychomotor impairing, and sedative properties. In contrast, ethanol's anxiolytic, anticonvulsant, amnestic and reinforcing properties have been studied less thoroughly, largely because tests for these effects are more difficult to develop. We are currently developing tests suitable for investigating these latter three properties of ethanol in mice. Following validation of the tests, we will investigate relationships between alcohol's various behavioral effects using a large number of inbred strains of mice which differ in their behavioral responses to alcohol. The results of these experiments should allow us to determine which behavioral effects of ethanol are related. Behavioral genetic techniques will then be applied to investigate the genetic basis for the differences in response to alcohol.

Alcohol shares many properties with drugs acting at the benzodiazepine-GABA receptor macromolecular complex. In order to determine which (if any) of its effects are mediated through this site, the interactions of alcohol with various ligands for the complex are being investigated in the behavioral tests.

Several lines of evidence suggest that learning and memory can be studied in a number of different forms. For example, a subject may appear to be amnesic when a test of episodic learning is used, but perform normally in a test of procedural learning. In the present series of studies we are attempting to determine which cognitive processes are distinct. We believe that cognitive dysfunction can yield information about normal cognitive functioning and are, therefore, comparing the performance of several populations of subjects in a battery of cognitive tests. The subjects we are testing include normal volunteers under the influence of either alcohol or a benzo-



diazepine and patients with various memory disorders including Korsakoff's disease, Alzheimer's disease and alcoholic dementia. By determining what these populations can and cannot learn and remember, we expect to provide evidence to support distinctions between various cognitive processes.

A similar approach is being used in laboratory rodents. We are assessing the performance of different populations of mice (inbred and outbred strains) in various tests of learning and memory, and also comparing the effects of various drugs known to alter learning and memory processes.

We are also investigating the effect of drug-induced alterations in mood on cognitive function. It is likely that ethanol is self-administered at least in part for its mood-elevating effects. Very little is known in this area, however, and what has been reported is generally anecdotal rather than experimental. We are examining changes in mood following the consumption of alcohol and examining how such changes alter several cognitive processes.

#### Methods Employed:

The plus-maze test of anxiety is used to assess anxiolytic effects of various drugs. Mice are placed on an elevated plus-maze consisting of two open and two closed arms. The proportion of time spent on the open arms expressed as a percentage of the total time on both types of arms, and the percentage of arm entries made into the open arms are used as two indices of anxiety. Animals are tested for 5 min in a holeboard apparatus immediately before the test to give measures of locomotion and directed exploration.

A conditioned-place preference paradigm is being developed for use in mice based upon previous work with rats, to assess the reinforcing properties of different drugs. A large number of different inbred strains of mice are being tested in the holeboard, on the plus-maze, and in the place-preference paradigm, and their response to alcohol, and to benzodiazepine-receptor ligands is being examined. The data gathered from these tests will be added to data already collected in the same strains by Dr. John Crabbe (VA, Portland, OR) on other behavioral effects of alcohol (hypnotic, hypothermic, and ataxic). It should be possible to determine which of ethanol's effects are related. A genetic analysis aimed at determining the number of loci responsible for strain differences is being performed by Dr. David Goldman.

To assess the interactions of ethanol with various drugs that act at the benzodiazepine-GABA receptor macromolecular complex three different paradigms are being used: the holeboard, the plus-maze, and a seizure threshold paradigm in which seizure threshold to the convulsant biculline is determined.

An extensive test of learning and memory has been developed for use in humans which assesses: episodic memory, procedural learning, several different forms

of priming, retrieval of information from semantic memory, and recognition memory. This test is being given to patients with Korsakoff's disease, Alzheimer's disease or alcoholic dementia. A shorter version is being used to investigate the acute effects of ethanol on these various memory functions.

The memory tests we are using in animals include: the habituation of exploratory behavior, assessed by repeatedly testing animals in the holeboard apparatus; the radial arm maze which is a spatial learning task that relies on both reference and working memory; a passive avoidance test.

In experiments investigating interactions between mood and cognition, normal volunteers are being studied. They are given a variety of tests to perform, and mood-ratings questionnaires to complete, in both a sober state and following the consumption of an alcohol-containing beverage, on the rising portion of the blood-alcohol curve. The battery of cognitive tests has been designed to assess the effects of mood on the encoding and retrieval of information of different affective tone. Both semantic and episodic memory function are assessed.

#### Major Findings:

The benzodiazepine receptor ligand Ro 15-4513 has been found to partially reverse the effects of ethanol in the seizure threshold paradigm and in the holeboard test. In addition to antagonising the effects of ethanol the compound possesses intrinsic behavioral effects of its own, reducing seizure threshold to bicuculline, pentylenetetrazole and Ro 15-3663. It also reduces exploratory head-dipping in the holeboard. It causes seizures in DBA/2 mice and is proconvulsant in mice undergoing ethanol withdrawal.

The antagonism is not selective for ethanol. In both the seizure threshold and holeboard tests Ro 15-4513 partially reversed the effects of sodium pentobarbital. In contrast to the partial antagonism of the effects of ethanol and the barbiturate, Ro 15-4513 was able to completely antagonise the behavioral effects of the benzodiazepine diazepam in both the seizure threshold and holeboard tests.

Another benzodiazepine receptor inverse agonist FG 7142 was also able to partially reverse the effects of ethanol in the holeboard.

The effects of ethanol in the holeboard test depends on the dose of ethanol, the strain of mouse under investigation, and whether or not the animals are familiar with the testing apparatus. In DBA/2 mice ethanol causes a dose related increase in locomotor activity, but decreases motor activity in C57BL/6 mice. In the DBA/2 mice, a low (0.8 g/kg) dose of ethanol increases exploratory head-dipping, but a high dose (2.4 g/kg) decreases this measure. Further, an increase in exploration following treatment with the lower dose is only observed if the animals have not previously been familiarized with the testing apparatus.

The locomotor stimulant action of ethanol can be potentiated by pairing a single dose of ethanol with the testing apparatus 48 hrs. earlier. This potentiation of ethanol's effect is not observed if animals received ethanol but no exposure to the test apparatus 48 hrs. earlier.

Significance to Biomedical Research and the Program of the Institute:

Alcoholism has been demonstrated to possess a significant genetic component. It is important, therefore, to develop animal models that not only measure processes that contribute to the development of alcohol dependence, but that are also amenable to genetic analysis. We believe that alcohol is more likely to be self-administered for its anxiolytic and reinforcing properties than for its hypnotic or hypothermic effects. Our current approach using the plus-maze and conditioned place preference tests will enable us to investigate the roles played both by the genes and environment in animals' sensitivity to the anxiolytic and reinforcing properties of alcohol.

It has been suggested that Ro 15-4513 or a similar compound may be useful clinically in treating alcoholics, or possibly by reducing the effects of acute alcohol intoxication. Our investigations into intrinsic properties of this compound indicate that it will have little clinical use in view of its proconvulsant effects, and its ability to induce seizures in animals undergoing ethanol withdrawal. Further, the antagonism of ethanol's effects is far from complete. While this compound may have little clinical use, it is of great benefit in understanding the neurochemical mechanisms underlying ethanol's behavioral effects, and emphasizes the importance of the benzodiazepine/GABA receptor complex in these effects.

The ability to distinguish between various basic processes involved in learning and memory is of fundamental importance both in diagnosis and in the treatment of memory disorders. We hope to identify equivalent processes in humans and laboratory animals using the present approach, and this will allow a more efficient screening of potential treatments.

Previous studies have shown that mood can have quite marked effects on the way information is retrieved and acquired. However, the methods used to induce different moods in these studies have been subject to criticism. The current studies circumvent a number of these problems by using drugs as the means of altering mood. If we are able to demonstrate that ethanol alters cognitive processing via its effects on mood, this may not only add to our understanding of why people drink, but will also provide the cognitive psychologist with a valuable method to further investigate how mood can affect cognition.

Proposed Course:

We shall continue gathering data using the tests outlined above. In our animal studies we intend to investigate some lines of wild mice bred at Hazelton Laboratories since they are likely to provide even greater genetic variation than the inbred strains currently being tested.

Publications:

Karanian, J., Yergey, J., Lister, R.G., D'Souza, N., Linnoila, M., and Salem, N., Jr.: Characterization of an automated apparatus for precise control of inhalation chamber ethanol vapor and blood ethanol levels. Alcoholism: Clin. Exp. Res. 10:443-447, 1986.

Lister, R.G., and Nutt, D.J.: Mice and rats are sensitized to the proconvulsant action of benzodiazepine-receptor inverse agonist (FG 7142) following a single dose of lorazepam. Brain Res. 379:364-366, 1986.

Lister, R.G.: The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology 92:180-185, 1987.

Lister, R.G.: The effects of repeated doses of ethanol on exploration and its habituation. Psychopharmacology 92:78-83, 1987.

Lister, R.G. and File, S.E.: The effect of chlordiazepoxide on the habituation of exploration: interactions with the benzodiazepine antagonist Ro 15-1788. Pharmacol. Biochem. Behav. 26:631-634, 1987.

Lister, R.G.: The effects of ethanol on exploration in DBA/2 and C57B1/6 mice. Alcohol 4:17-19, 1987.

Lister, R.G., LeDuc, B.W., Greenblatt, D.J. and File, S.E.: Poor bioavailability of CGS 8216 in a water/tween vehicle following intraperitoneal injection. Psychopharmacology 91:260-261, 1987.

Lister, R.G. and Nutt, D.J.: Is Ro 15-4513 a specific alcohol antagonist? Trends Neurosci. 10:223-225, 1987.

Nutt, D.J. and Lister, R.G.: The effect of the imidazodiazepine Ro 15-4513 on the anticonvulsant effects of diazepam, sodium pentobarbital and ethanol. Brain Res. (in press).

Lister, R.G.: Interactions of Ro 15-4513 with diazepam, sodium pentobarbital and ethanol in a holeboard test. Pharmacol. Biochem. Behav. (in press).

Lister, R.G.: Reversal of the intrinsic effects of Ro 15-4513 on exploratory behavior by two benzodiazepine receptor antagonists. Neurosci Letters. (in press).

Lister, R.G.: The benzodiazepine receptor inverse agonists FG 7142 and Ro 15-4513 both reverse some of the behavioral effects of ethanol in a holeboard test. Life Sci. (in press).

Lister, R.G. and Karanian, J.: Ro 15-4513 induces seizures in DBA/2 mice undergoing ethanol withdrawal. Alcohol. (in press).

Goldman, D.J., Crabbe, J.C. and Lister, R.G.: Mapping of a putative genetic locus determining ethanol intake in the mouse. Brain Res. (in press).

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00250-04 LCS
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Electrophysiological Studies of Acute and Chronic Alcohol Consumption		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	J. Rohrbaugh	Research Psychologist LCS, NIAAA
Others:	B. Adinoff	Senior Staff Fellow LCS, NIAAA
	M. Eckardt	Section Chief LCS, NIAAA
	J. Johnson	Research Psychologist LCS, NIAAA
	M. Linnoila	Chief LCS, NIAAA
	D. Rio	Physicist LCS, NIAAA
	J. Stapleton	Staff Fellow LCS, NIAAA
COOPERATING UNITS (if any) Department of Psychology, Catholic University (R. Parasuraman); Department of Electrical Engineering, University of Nebraska (J. Varner)		
LAB/BRANCH Laboratory of Clinical Studies		
SECTION Section of Clinical Brain Research		
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Although alcohol clearly leads to alterations in mental processes and behavior, it is perhaps because these effects are so great and so pervasive that they have proven difficult to characterize. The global performance measures commonly used to document such effects, while demonstrating clearly their presence, often provide little information as to the specific locus or mechanism of effect. The present research aims to parse the separate effects of alcohol on peripheral nerve, sensory, cognitive and motor systems using event related brain electrical potentials. These potentials are studied within a broad context provided by performance, psychophysiological, neuropsychological, neuro-radiological and neuropsychiatric data.</p> <p>The effects of alcohol are investigated in three classes of subjects: 1) The effects of acute administration are examined in normal volunteers with respect to dose-response relationships, the temporal course of effect, and relationship to blood alcohol levels; 2) Brain electrical activity is studied, in conjunction with neuropsychological data, in social drinkers in an attempt to assay the effects of moderate levels of alcohol consumption; 3) Abstaining alcoholics, including those with alcoholism-related mental impairment, are studied to characterize the deficits, to examine the short- and long-term recovery effects, if any, during abstinence, and to examine the efficacy of various treatment strategies.</p>		

PROJECT DESCRIPTION:Investigators:

J. Rohrbaugh	Research Psychologist	LCS, NIAAA
B. Adinoff	Senior Staff Fellow	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
J. Johnson	Research Psychologist	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
D. Rio	Physicist	LCS, NIAAA
J. Stapleton	Staff Fellow	LCS, NIAAA
J. Varner	Associate Professor	Univ. of Nebraska
R. Parasuraman	Associate Professor	Catholic Univ.

Objectives:

This research aims to provide a comprehensive overview of the effects of alcohol, both acute and chronic, on sensory, cognitive and motor systems. A primary focus will be upon event-related brain electrical potentials, elicited in response to environmental stimulation, and extracted by computer from the on-going EEG. The electrical responses will be studied in various tasks and under various conditions for information about the related neural processes and possible disturbances associated with alcohol consumption. Measurement of the brain electrical potentials will be accompanied by simultaneous measurement of psychophysical judgments, reaction time and other behavioral responses, as well as responses in autonomic and somatic systems (EKG, electrodermal, electromyographic, pupillary, vasomotor, respiratory and oculomotor responses).

Methods Employed:

Brain electrical activity and psychophysiological responses are measured from surface electrodes using conventional EEG and polygraph instruments, allowing data to be acquired simultaneously from as many as 40 channels. The responses are analyzed with respect to waveform and sensitivity to experimental variables using multivariate techniques. Topographic distributions of the responses over the scalp are studied for evidence of neural sources of the electrical activity, using scalp mapping and dipole inference techniques.

Sensory functions are evaluated separately for visual, acoustic and somatosensory systems using clinically validated techniques. Visual stimulation and recording techniques permit evaluation of function in retinal, optic nerve and tract, and cortical centers. Similarly, auditory and somatosensory techniques permit examination of function in peripheral, brainstem and cortical areas. Cognitive function is assessed by examination of responses that are related to attention and decision making as described representatively below:

1) Vigilance and habituation. Habituation of response amplitude or frequency upon repetitive stimulation is a primitive and ubiquitous form of behavioral plasticity that has been proposed to underlie a variety of complex learning and performance abilities. Data indicate that alcohol affects this process, as measured by a number of ERP, autonomic and behavioral measures. The decline in

responsiveness is particularly evident in sustained attention, or vigilance, situations. We are further investigating these changes in a number of paradigms, including traditional habituation, dishabituation and spontaneous recovery paradigms in which autonomic and ERP measures are studied. Performance measures are derived from a vigilance task that has been developed to show a particularly rapid rate of performance decrement, and thus allow the acute effects of alcohol to be studied separately at ascending and descending limbs of the blood alcohol curve. This vigilance task also permits separate examination of sensitivity and report criterion changes during the time of task.

2) Orienting. The orienting response is generally conceived as a transient attentional response to novel or significant stimuli. Although the response habituates rapidly to innocuous stimuli, some data indicate that the response to task-relevant stimuli may persist so long as salience is maintained. The response is believed to be generally important in a variety of memory, conditioning and cognitive skills. In our studies of the effects of alcohol on orienting, we are concentrating particularly on a slow, late component of the ERP (the O wave), which we believe to be a central manifestation of orienting. The evidence for this belief includes observations that the eliciting conditions are appropriate, i.e. the O wave appears only in response to stimuli that are greatly discrepant from expected stimuli, or that are task relevant and significant. The wave is accompanied by autonomic responses that have more traditionally served to earmark orienting responses, and its temporal course is similar to that shown by performance measures of orienting. Moreover, it appears to originate in a right dorsolateral frontal area that is believed to be particularly important in the regulation of attention. The right frontal origins of the O wave suggest to us that its study is likely to be especially fruitful in light of hypotheses that the effects of alcohol selectively target functions subserved by frontal and/or right hemisphere cortices.

3) Effects of Attention on Sensory Systems. A long-favored strategy for studying attention is to examine responses to probe stimuli introduced during behaviorally different periods. We have developed an elaboration of this strategy that allows the extent, distribution across sensory modalities or channels, and temporal course of attentional allocation to be traced continuously. The probe stimuli in this technique are weak, background stimuli that are repetitively presented at a steady rate. Under such conditions, a steady EEG rhythm is established, which is believed to be composed of early responses in primary sensory cortices. Our technique is to establish this rhythm under conditions demanding various forms of attention, and to extract the rhythm from the composite EEG using digital filtering techniques for continuous examination of phase and amplitude. Studies from normal subjects indicate that the nature of this rhythm varies in a systematic and theoretically consistent manner; current studies are using this response to infer the appropriateness and extent of attention as influenced by the acute and chronic effects of alcohol.

4) Information Processing Stages. A powerful method, we believe, for identifying specific loci of alcohol's effects upon performance is presented by the additive factor method, in which information processing is presumed to involve a series of sequential stages. By manipulation of such features as stimulus in-

tensity, clarity or complexity, and assessing the effects on speeded response performance, the stages disturbed by alcohol can be inferred. Examination of ERP components within this context provides convergent evidence. Particularly relevant is the "P300" component, which is a positive component peaking at latencies of 300 msec or greater post stimulus. Previous experiments have suggested that P300 is particularly important in light of data suggesting that P300 is diminished in chronic alcoholics and in their offspring, as well.

#### Major Findings:

During the period covered in this report, additional data have been obtained from selected patients diagnosed as suffering from alcohol-related organic brain syndromes, primarily Korsakoff's disease. Fourteen patients have been studied intensively under baseline conditions and within the context of an ongoing experiment that examines the chronic effects of the serotonin reuptake inhibitor fluvoxamine. For comparison purposes, data have been obtained from ten age-matched normal subjects as well. Subjects participate in a variety of tests of sensory, brainstem and cortical function. Preliminary analyses reveal differences between normal subjects and patients in the P300 component, but no appreciable effect of fluvoxamine. This latter finding is in accord with evidence that P300 is generated or mediated primarily by cholinergic systems.

We have also completed a large-scale dose response study of the acute effects of alcohol on information processing and ERPs. Data were obtained from 12 nonalcoholic males who had received four doses. Three tasks were investigated. These tasks were designed to illuminate processes related to stimulus encoding and identification, movement selection and execution, and vigilance. Performance data indicate that the effects of alcohol are quite specific, insofar as alcohol appears to spare a number of specific information processing stages related to identification, decision and response selection. Alcohol has appreciable effects on ERP components related to stimulus encoding, however, suggesting that this stage of information processing is adversely affected.

The acute effects of alcohol on the motor system were studied in a situation in which skilled movements were performed. The signal to make the movement was forewarned with a constant period of 4 sec. ERPs measured in this situation provide no evidence that alcohol diminished the effectiveness of the warning signal; if anything, responses to it were somewhat greater with moderate and high doses. The ERP potentials related to preparation for movement, however, were altered by alcohol. Whereas alcohol had little effect on potentials recorded over the primary motor sites, it had appreciable effects on the distribution of the potentials over the head. Anterior and posterior activity was disproportionately reduced. This effect may represent a diminished contribution from frontal and posterior areas that are important for planning and regulation of movement.

Appreciable effects of alcohol were also found on sustained attention (i.e., vigilance). The vigilance task entailed monitoring a continuous stream of degraded visual stimuli for occasional target stimuli. Performance in this



task typically declines over a period of several minutes. Data obtained show for the first time that alcohol adversely affects the rate of performance decline, i.e., whereas the detrimental effects of alcohol may at first be small or indiscernible, they become exaggerated over time. This demonstration has both theoretical and practical significance. On a theoretical level, it indicates that alcohol affects the availability of cognitive processing capacity and the ability to maintain it over time. On a practical level, it has relevance to the interpretation of accident statistics for driving and other tasks requiring continuous performance. Moreover, the finding that alcohol's effects are exacerbated with time on task has implications for the translation of discrete laboratory tasks to field situations in which performance demands are more likely to be continuous.

Data collection has begun on a major topographic distribution study which will investigate the relationship of the steady EEG rhythm we have been studying to midlatency sensory responses as well as later components (O wave and P300) by comparing topographic characteristics in the same subjects. Data will also be used to further develop strategies for equivalent dipole modelling in order to explore commonalities in underlying electromotive generators.

#### Significance to Biomedical Research and the Program of the Institute:

These studies offer the prospect of characterizing more completely the extent and nature of the effects of alcohol on the nervous system. This information will be valuable in describing and accounting for the detrimental effects of alcohol on performance, and will in addition be of diagnostic and prognostic value in patients suffering effects from chronic alcohol consumption.

#### Proposed Course:

Immediate plans are to complete analyses of the acute and chronic studies described above, and to complete data collection on the topographic mapping study. Continued emphasis will be placed on the development of paradigms and recording procedures that are selectively responsive to the effects of alcohol on various neural systems.

#### Publications:

Rohrbaugh, J.W., Stapleton, J.M., Parasuraman, R., Frowein, H., Eckardt, M.J. and Linnoila, M.: Alcohol intoxication in humans: Effects on vigilance performance. Alcohol and Alcoholism, Suppl. 1:97-102, 1987.

Rohrbaugh, J.W., Stapleton, J.M., Parasuraman, R., Zubovic, E., Frowein, H.W., Eckardt, M.J., and Linnoila, M.: Dose-related effects of alcohol in a sustained attention task. In Rohrbaugh, J.W., and Begleiter, H. (Eds.): Event-Related Brain Potentials and Alcohol. Alcohol (special issue) (in press).

Rohrbaugh, J.W., Birbaumer, N., Gaillard, A., McCallum, W.C., Papakostopoulos, D., Simons, R.F.: Event-related potentials associated with preparatory and movement-related processes. In McCallum, W.C., Zappoli, R. and Denoth, F. (Eds.): Cerebral Psychophysiology: Studies in Event-Related Potentials. Amsterdam, Elsevier, 189-229, 1986.

Rawlings, R.R., Rohrbaugh, J.W., Begleiter, H., and Eckardt, M.J.: Spectral methods for principal components analysis of event-related brain potentials. Computers in Biomedical Research 19:497-507, 1986.

Rohrbaugh, J.W., Johnson, R., and Parasuraman, R. (Eds.): Event-Related Brain Potentials: Issues and Interdisciplinary Vantages. New York, Oxford University Press (in press).

Johnson, R., Rohrbaugh, J., and Parasuraman, R. (Eds.): Current Trends in ERP Research. Amsterdam, Elsevier (in press).

Rohrbaugh, J.W. and Begleiter, H. (Eds): Event-Related Brain Potentials and Alcohol. Alcohol (special issue) (in press).

Stapleton, J.M., Guthrie, S., and Linnoila, M.: Effects of alcohol and other psychotropic drugs on eye movements: Relevance to traffic safety. Journal of Studies on Alcohol 47:426-432, 1986.

Rohrbaugh, J.W., Varner, J.L., Peters, J.F., Ellingson, R.J. and Eckardt, M.J.: Brainstem auditory evoked potentials are unaltered during the orienting response. In: R. Johnson, Jr., J.W. Rohrbaugh and R. Parasuraman (Eds.) Current Trends in ERP Research. Amsterdam: Elsevier, (in press).

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00237-05 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Individual Variability in Drug Metabolism by Carbon Dioxide Breath Tests

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E. Lane	Senior Staff Fellow	LCS, NIAAA
Others:	I. Parashos	Visiting Fellow	LCS, NIAAA
	N. Carson	Biologist	LCS, NIAAA
	M. Towle	Chemist	LCS, NIAAA
	B. Ravitz	Medical Staff Fellow	LCS, NIAAA

## COOPERATING UNITS (if any)

Epilepsy Branch, NINCDS (R. Porter); Nursing Dept., NINCDS (I. Naveau)

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Biochemistry and Pharmacology

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS

2.9

## PROFESSIONAL:

0.9

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Preliminary testing of the prediction that caffeine, a low extraction ratio drug (0.1) should be a more sensitive probe of enzyme induction than methacetin, a high extraction ratio drug (0.9), when excretion of a metabolite (CO<sub>2</sub>) is measured, has been carried out. A single dose of each was administered to 8 healthy volunteers and 9 epilepsy patients treated with phenytoin, carbamazepine and/or phenobarbital. The <sup>13</sup>C carbon dioxide in expired breath was measured by isotope ratio mass spectrometry. The percentages of the dose excreted as CO<sub>2</sub> in 2 hr. were compared: 3.22% ± 0.86 and 5.54% ± 1.59 caffeine was excreted by controls and patients, respectively, compared with 28.6% ± 5.8 and 40.0% ± 4.2 methacetin. The results in the 2 subject groups were significantly different for both probes (p<.05). These data do not support the theoretical prediction that the extraction ratio of a drug has a critical effect upon its usefulness in detection of induction of oxidative metabolism via the carbon dioxide breath test.

The pharmacokinetics of ethanol have been studied by measurement of blood ethanol concentrations during and after four oral dosing rates and one intravenous infusion to four healthy volunteers. The consumption of breakfast after two hours of oral dosing disrupted the smooth approach to steady state concentrations which cannot be explained as a simple delay in absorption. The data indicate a significant effect of food upon the bioavailability of ethanol probably due to increase in stomach emptying time.

PROJECT DESCRIPTION:Investigators:

E. Lane	Staff Fellow	LCS, NIAAA
I. Parashos	Visiting Fellow	LCS, NIAAA
N. Carson	Biologist	LCS, NIAAA
M. Towle	Chemist	LCS, NIAAA
B. Ravitz	Medical Staff Fellow	LCS, NIAAA
R. Porter	Chief	MNB, NINCDS
I. Naveau	Clinical Nurse	Nursing Dept., NINCDS

Objectives:

This project is designed to use a low risk and relatively noninvasive technique to evaluate the rate at which an individual patient can metabolize drugs.

Methods Employed:

The test drugs are labeled with a stable isotope of carbon ( $^{13}\text{C}$ ) in a methyl group that is removed by liver enzymes. This labeled carbon then appears in expired carbon dioxide where it can be measured, using an isotope ratio mass spectrometer. Predictions of the suitability of particular drugs as probes for drug metabolizing ability are made using pharmacokinetic models and computer simulations. The test drug and the complimentary metabolite formed when the test drug is demethylated are measured in plasma by means of high performance liquid chromatography.

Major Findings:

The percentage of a dose of either a low (caffeine) or high (methacetin) extraction ratio drug excreted as  $\text{CO}_2$  within 2 hr. of a dose was measured in a small sample of drug free healthy volunteers and epilepsy patients taking enzyme inducing anticonvulsants. The results were statistically significantly different for both probes, therefore, failing to support the theoretical prediction that the extraction ratio of a drug has a critical effect upon its usefulness in detection of induction of oxidative metabolism via measurement of excretion of the metabolite  $\text{CO}_2$ .

Significance to Biomedical Research and the Program of the Institute:

Many factors, including alcohol consumption, affect the ability of an individual to metabolize various drugs. A method, such as this breath test, for rapid evaluation of drug metabolizing ability, should improve individualization of drug treatments. This would have particular application to times when drug metabolizing ability is changing because of alcohol withdrawal and treatment of alcoholism.

Proposed Course:

Evaluation of this test in alcoholics, before and during treatment and in epileptic patients during treatment with enzyme inducing drugs, is proposed. Drugs previously used (aminopyrine, caffeine and methacetin) and another drug (imipramine) will be evaluated as probes in this liver function test.

Publications:

Guthrie, S., and Lane, E.A.: A pharmacokinetic analysis of the oral benzo-diazepine ethanol interaction. Alcoholism: Clin. and Exper. Res. 10:686-690, (1986).

Insel, T.R., Lane, E.A., Sheinin, M., and Linnoila, M.: Acute and chronic effects of desipramine administration to rhesus monkeys. Eur. J. Pharmac. 136:63-68, (1987).



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00248-04 LCS
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Acetylation Phenotype of Alcoholics		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <span>PI: E. Lane</span> <span>Senior Staff Fellow</span> <span>LCS, NIAAA</span> </div>		
COOPERATING UNITS (if any) <div style="height: 40px; margin-top: 10px;">None</div>		
LAB/BRANCH Laboratory of Clinical Studies		
SECTION Section of Clinical Biochemistry and Pharmacology		
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892		
TOTAL MAN-YEARS: 0.1	PROFESSIONAL: 0.1	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div> <input checked="" type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div> <input type="checkbox"/> (b) Human tissues         </div> <div> <input type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Any coincidence of the slow acetylator phenotype and alcoholism will be investigated in this study.  The proportion of alcoholic subjects that exhibits the slow acetylator phenotype is to be compared with the proportion of normal volunteers that exhibits the slow acetylator phenotype.  The acetylator phenotype will be measured in normal volunteers and recovered alcoholics, who are not related to each other. The recovered alcoholics should have a history of alcoholism and the normal volunteers should be nonalcoholic and have no first degree relatives who are alcoholic.  The acetylator phenotype will be determined after a single dose of sulfamethazine by measurement of blood and urine concentrations of sulfamethazine and its acetylated metabolite. These will be quantified by high pressure liquid chromatography.  The acetylator phenotype has been determined in a total of 38 alcoholics and 28 unrelated normal volunteers. This number of subjects is insufficient to draw any conclusions regarding the relative distribution of the slow acetylator phenotype in the two populations.		

PROJECT DESCRIPTION

Investigators:

E. Lane

Senior Staff Fellow

LCS, NIAAA

Objectives:

Any coincidence of the slow acetylator phenotype and alcoholism will be investigated in this study.

Methods Employed:

Acetylator phenotype is measured in unrelated, age and sex matched, normal volunteers and recovered alcoholics. The recovered alcoholics should have a history of alcoholism and the normal volunteers are nonalcoholics and have no first degree relatives who are alcoholic.

The acetylator phenotype will be determined after a single dose of sulfamethazine by measurement of blood and urine concentrations of sulfamethazine and its acetylated metabolite. These will be quantified by high pressure liquid chromatography.

Major Findings:

There are no findings at this stage.

Significance to Biomedical Research and the Program of the Insititute:

There are two possible points of association between acetylation phenotype and alcoholism. (1) Recent alcohol consumption can alter the "apparent" acetylator phenotype of an individual by increasing his/her acetylation clearance. (2) Acetylation phenotype is, and alcoholism may be, genetically determined and the expression of alcoholism may depend upon genetic factors related to acetylation.

If there should be any coincidence of acetylator phenotype and alcoholism, then further avenues of research in alcoholism will be undertaken.

Publications:

None.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00255-03 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Application of Pharmacokinetics to Neurotransmitter Disposition

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: E. Lane Senior Staff Fellow LCS, NIAAA

Others: I. Parashos Visiting Fellow LCS, NIAAA  
M. Linnoila Chief LCS, NIAAA

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Clinical Biochemistry and Pharmacology

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A model for the disposition of centrally and peripherally produced norepinephrine has been used to design experiments to separately quantify the central and peripheral production rates of norepinephrine in vivo. Analytical methods required for the execution of these studies have been established in this laboratory. These include the measurement of norepinephrine, normetanephrine, 3-methoxy-4-hydroxy phenylglycol and vanilmandelic acid by gas chromatography-mass spectrometry. Preliminary experiments in animals have demonstrated that some of the analytical methods must be further refined in order to serve our experimental design.

This project incorporates Z01 AA 00246-04.

PROJECT DESCRIPTION:

Investigators:

E. Lane	Senior Staff Fellow	LCS, NIAAA
I. Parashos	Visiting Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA

Objectives:

Studies are designed to elucidate the in vivo disposition of neurotransmitters and their metabolites in animals and man and the effects of various treatments upon that disposition. This may lead to new hypotheses about the biochemical bases of psychiatric disorders such as alcoholism and the mechanisms of action of drugs used in their treatment.

Methods Employed:

The disposition of neurotransmitters and their metabolites are modeled according to available knowledge including that of metabolic pathways and the sites of metabolism. Studies are then designed to fill in the unknown factors of the model. These studies include administration of neurotransmitters and metabolites to animals and man and analyses of these compounds and metabolites in various biological fluids. The administered substances are usually labeled with radioactive or stable isotopes. The analyses are accomplished by high pressure liquid chromatography, gas-liquid chromatography and mass spectrometry.

Major Findings:

There are no major findings this year.

Significance to Biomedical Research and the Program of the Institute:

Rational interpretation of in vivo measurements of neurotransmitters and their metabolites will help clarify any neurochemical aspects of alcoholism.

Proposed Course:

We are designing and carrying out studies to determine what fraction of the total daily production of the norepinephrine is derived from the central nervous system. When these methods have been tested on animal models they should be applicable to human subjects.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00235-05 LCS
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Metabolic and Structural Studies of Polyunsaturated Lipids in Cell Membranes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: N. Salem, Jr.                      Section Chief                      LCS, NIAAA		
Others: J. Yergey                      Senior Staff Fellow                      LCS, NIAAA J. Karanian                    Senior Staff Fellow                      LCS, NIAAA H. Kim                            Staff Fellow                              LCS, NIAAA T. Shingu                        Visiting Fellow                          LCS, NIAAA F. Hullin                        Visiting Fellow                          LCS, NIAAA A. Yoffe                            Chemist                                    LCS, NIAAA		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Clinical Studies		
SECTION Section of Analytical Chemistry		
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892		
TOTAL MAN-YEARS: 4.9	PROFESSIONAL: 3.0	OTHER: 1.9
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The principal objective of this study is to elucidate the structural and metabolic functions of polyunsaturated fatty acids or phospholipids with particular reference to their modulation by ethanol. Several approaches to this problem were taken including studies of cellular lipid composition, membrane asymmetry, fatty acid oxygenation and dietary supplementation. In particular, these studies focused on the major polyunsaturate of brain, docosahexaenoate (C22:6w3) and, to a lesser extent, on arachidonate (C20:4w6).</p> <p>The characterization of the enzymatic oxygenation of docosahexaenoate (22:6w3) was further characterized as a lipoxygenase system. Rat brain homogenate enzyme was sensitive to the lipoxygenase inhibitors ETYA, NDGA and caffeic acid but insensitive to cyclooxygenase inhibitors such as indomethacin. Preliminary results indicate that monohydroxylated-22:6 derivatives are formed by rat brain in vivo. This is the first demonstration of a 22:6 lipoxygenase product formed in vivo in any mammalian organism and is therefore of general significance. A second avenue of investigation of the biological effects of polyunsaturates involved the effects of the 22:6 fatty acid on aortic contractility. It was observed that micromolar concentrations of 22:6 produced a decrease in muscle tone in the rat aorta and that this effect does not appear to be mediated by prostaglandins or leukotriene formation.</p> <p>Continued development of thermospray liquid chromatographic/mass spectrometric (LC/MS) techniques for the analysis of phospholipid species has made possible a rapid, detailed and efficient analysis of all of the major lipid classes. Progress has been made in the development of quantitative methods and initial results of ethanol exposure on biological tissue lipid composition is presented.</p>		

PROJECT DESCRIPTION:Investigators:

N. Salem, Jr.	Section Chief	LCS, NIAAA
J. Karanian	Senior Staff Fellow	LCS, NIAAA
J. Yergey	Senior Staff Fellow	LCS, NIAAA
H. Kim	Staff Fellow	LCS, NIAAA
T. Shingu	Visiting Fellow	LCS, NIAAA
F. Hullin	Visiting Fellow	LCS, NIAAA
A. Yoffe	Chemist	LCS, NIAAA
M.B. Engler	Graduate Student	Georgetown Univ.

Objectives:

(1) In general, to enumerate the various functions of polyunsaturated lipids particularly with reference to excitable membranes and to the chemical transfer of information between cells, with the objective of applying the information obtained to a better understanding of the mechanism of action of ethanol. (2) To elucidate the molecular composition, associations and topographic arrangement of polyunsaturated phospholipids in the plasma membrane. (3) To discover and characterize a novel metabolic system of lipooxygenase and/or cyclooxygenase enzymes in the mammalian brain which operates on W-3 fatty acids.

Methods Employed:

For oxygenated metabolism studies of 22:6w3, <sup>14</sup>C-labelled fatty acid (0.15mM) was incubated for 15 min at 37°C with rat brain homogenate or platelets. The solution was acidified to pH 3.5, extracted into dichloromethane, concentrated, filtered and injected into a reversed-phase HPLC system. Fractions thus produced were monitored for radioactivity using a Radiomatic Model IC Flow detector and data processing system.

Thermospray LC/MS was performed on an Extrel 400-2 quadrupole mass spectrometer using a Vestec interface. The vaporizer tip and source temperatures were 145 and 300°C, respectively and ionization assistance was achieved by applying an electron-emitting filament current.

Lipids were extracted using the method of Bligh and Dyer from brain, heart, liver, and red blood cells and samples transmethylated with boron trifluoride in methanol. Gas chromatographic analyses of the methyl esters were performed by GC/FID or GC/MS using an OV-351 capillary column. Similar studies were also performed on rat tissues after 1-14 days of exposure to ethanol vapors.

Major Findings

Great impetus has been provided to the study of 22:6w3 enzymatic oxygenation in the brain by the use of other preparations with defined lipooxygenase activities. Purified soybean 15-lipooxygenase was used to generate 17-hydroxy-22:6w3 and human platelets were used to generate 14-hydroxy-22:6. An HPLC

separation technique was evolved for separating these docosanoids using an Altex ODS column (0.46x25cm, 5um), a flow rate of 1 ml/min, a solvent system of 100mM ammonium acetate, pH 6.0 versus acetonitrile (solvent B) and the following gradient:

<u>Time (min)</u>	<u>% B</u>
0	25
20	40
40	45
60	50
80	100
90	100
100	25

The 17-hydroxy- and 14-hydroxy-22:6 standards generated by soybean and platelet enzymes chromatographed with retention times of 38 and 37 min, respectively. Peaks with corresponding retention times were observed in rat brain homogenate enzyme in vitro and also in rat brain in vivo after a 10 uCi  $^{14}\text{C}$ -22:6w3 injection into the lateral ventricle. Production of these compounds in vitro was prevented by the addition of the lipoxygenase inhibitors NDGA or ETYA. Production of a further peak running at 42 min. was sensitive to the 5-lipoxygenase inhibitor caffeic acid and was presumed to be 7-hydroxy-22:6. Rat brain homogenate production of these 22:6 metabolites was stimulated by ferrous iron or lowering pH (e.g., to 6.7) but these effects were not observed for boiled homogenate indicating an enzymatic basis for their conversion. The platelet enzyme was characterized as follows: production varied with the number of platelets and incubation time up until 20 min and was inhibited by boiling or by 10 uM ETYA but was stimulated by more than 1% ethanol. Human platelets could be used up until four days after drawing.

A second area of investigation was the possible direct action of 22:6 fatty acid on smooth muscle contraction. A concentration dependent relaxation of rat aortic strips was observed in the 1-64 uM range when the sodium salt of 22:6 was added to the baths. Indomethacin and NDGA preincubation had no effect on this phenomenon ruling out eicosanoids as mediators of the 22:6 effect on muscle tone.

Further development of the thermospray LC/MS approach to phospholipid molecular species analysis was principally in the area of quantification method development. Calibration curves of amount versus area of various phosphatidylcholine species indicated linearity but with differing response factors. Calibration curves using internal standards showed linear response in the 0.1-10 nmole range HPLC separation. Partial synthesis of some deuterated phosphatidylcholine species was performed in order to generalize this approach to more unsaturated species for application to analysis of alcoholic red blood cell polyunsaturates. These methods were applied to liver microsomal phospholipids after ethanol exposure and variations in the distributions of species was observed. There was generally a decrease in 20:4w6 and an increase in other W-6 species in liver PI. Fatty acid analysis of liver phospholipid classes after ethanol inhalation showed a dramatic decline in 20:4w6 to a level less than half that of control in PS, PC and PI, but only a mild decline in PE. Plasmalogen and glycolipid spectra were characterized by LC/MS in this reporting period.

Significance to Biomedical Research and the Program of the Institute:

The discovery of a leukotriene-like lipooxygenase product formed by rat brain in vivo is a significant development for the elucidation of the functions of polyunsaturates in brain. This is the first description of 22:6 metabolism in vivo in any mammalian tissue. The demonstration of the modification of this pathway by alcohol exposure in vivo may allow confirmation of a general theory of alcohol action on polyunsaturate - containing microenvironments on the interior of the synaptic membrane. Continued development of thermospray LC/MS methods for phospholipid species quantification should greatly facilitate clinical analyses of compositional, membrane microenvironment and lipid metabolic alterations induced by alcohol. They should therefore be of use not only for alcohol researchers but for all lipid chemists.

Proposed Course:

We will continue our characterization of membrane microenvironments with respect to polyunsaturate localization and the effects of alcohol on these environments. Human red blood cell samples will be analyzed to establish whether alcoholics are abnormal in this respect. Nutritional studies will be extended to determine the effects of omega-3 fatty acids in various tissues with respect to both composition and localization and to determine whether the effects of alcohol can be reversed by such treatment. Thermospray LC/MS studies will undergo further basic development in order to develop facile methods for quantification of molecular species composition in complex biological samples. Docosahexaenoate metabolism studies in rat brain in vivo will be extended to determine whether alcohol inhalation alters the rate or route of metabolism. A sensitive GC/MS assay which can determine picogram amounts of hydroxylated - 22:6 metabolites will be developed for application to both animal and human tissues and fluids such as cerebrospinal fluid.

Publications:

Salem, Jr., N., Yoffe, A., Kim, H.Y., Karanian, J.W., and Taraschi: Effects of fish oils and alcohol on polyunsaturated lipids in membranes. In Lands, W.EM. (Ed.): Polyunsaturated Fatty Acids and Eicosanoids. (in press).

Salem, Jr., N., Kim, H.Y. and Yergey, J.A.: Thermospray-liquid chromatography/mass spectrometry of eicosanoids and phospholipids. Interm Symposia Series (in press).

Kim, H.Y., Yergey, J.A., and Salem, Jr., N.: Determination of eicosanoids, phospholipids and related compounds by thermospray LC/MS. J. Chromatogr. 394:155-170, 1987.

Shingu, T. and Salem, Jr., N.: Role of oxygen radicals in peroxidation of docosahexaenoic acid by rat brain homogenate in vitro. In Walden, Jr., T.L. and Hughes, H.N. (Eds): Prostaglandins and Lipid Metabolism in Radiation Injury. New York, Plenum Press, (in press).

Kim, H.Y., Salem, Jr., N.: Application of thermospray high performance liquid chromatography/mass spectrometry for determination of phospholipids and related compounds. Anal. Chem. 59:722-726, 1987.

Simopoulos, A.P. and Salem, Jr., N.: Purselane: a terrestrial source of omega-3 fatty acids. New Engl. J. Med. 315: 833, 1986.

Salem, Jr., N., H.Y. Kim, J. Yergey. Docosahexaenoic acid: membrane function and metabolism. In Simopoulos, A.P. and Kifer, R.R. (Eds.): The health effects of polyunsaturated fatty acids in seafoods. New York, Academic Press, 1986.

Simopoulos, A.P., Salem, Jr., N.: Letter to the Editor. New Eng. J. Med. 316:627, 1987.

Palmblad, J., Wannemacher, R.W., Salem, Jr., N., and D.G. Wright. Essential fatty acid deficiency and neutrophil function: studies of lipid-free total parental nutrition in monkeys. J. Immunology. (in press).

Doherty, J.D., Lauter, C.J. and Salem, Jr., N.: Synaptic effects of the synthetic pyrethroid resmethrin in rat brain in vitro. Comp. Biochem. Physiol. 84C:373-379, 1986.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00251-04 LCS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of Prostaglandins in Mediating the Effects of Alcohol on Smooth Muscle.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Salem, Jr.	Section Chief	LCS, NIAAA
Others:	J. Karanian	Senior Staff Fellow	LCS, NIAAA
	T. Shingu	Visiting Fellow	LCS, NIAAA
	A. Yoffe	Chemist	LCS, NIAAA

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Clinical Studies

## SECTION

Section of Analytical Chemistry

## INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.9

## PROFESSIONAL:

0.8

## OTHER:

1.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Ethanol-induced changes in blood pressure, vasoreactivity and platelet aggregation may have common etiologies which include the disruption of prostaglandin metabolism and catecholamine secretion. Ethanol has been shown to markedly alter w-6 fatty acid levels, prostaglandin metabolism, and catecholamine levels in the cardiovascular system. Prostaglandins such as prostacyclin (PGI<sub>2</sub>) and thromboxane (TXA<sub>2</sub>), and also catecholamines such as epinephrine (E) and norepinephrine (NE) are active in the cardiovascular system and may play a role in the pathophysiology associated with ethanol consumption. Using our automated apparatus for precise control of inhalation chamber ethanol vapor and blood ethanol concentrations, the effects of a range of ethanol doses on vascular tone and reactivity, platelet aggregation, fatty acid content, prostaglandin production and plasma catecholamine levels were determined in the rat. Acute exposure to moderate blood ethanol concentrations (BEC) was associated with increased vascular production of PGI<sub>2</sub> and plasma catecholamines, whereas platelet production of TXA<sub>2</sub> decreased. Blood pressure is elevated in these animals, however, platelet aggregation and the pressor effect of norepinephrine (NE) were mildly decreased. Chronic exposure to high BEC is associated with a dramatic reduction in vascular and platelet prostaglandin (PG) production and a slight increase in plasma catecholamine levels. Concentrations of fatty acid precursors to PGs were reduced by as much as 50% in the lipid extracts of these preparations. Blood pressure is generally reduced in these animals and platelet aggregation decreases dramatically, however, the pressor effects of TXA<sub>2</sub> and NE were significantly increased. Long-term ethanol exposure did not induce a reduction in fatty acid precursors of PGs or the capacity to produce 2-series PG in animals fed a diet rich in gamma-linolenic acid (20:3).

PROJECT DESCRIPTION:Investigators:

N. Salem, Jr.	Section Chief	LCS, NIAAA
J. Karanian	Senior Staff Fellow	LCS, NIAAA
T. Shingu	Visiting Fellow	LCS, NIAAA
A. Yoffe	Chemist	LCS, NIAAA
M.M. Engler	Graduate Student	Georgetown Univ.

Objectives:

To evaluate the modulatory effects of ethanol on the physiological functions of eicosanoids, neurohumoral substances and other bioactive compounds with particular reference to the cardiovascular system. In this reporting period, vascular smooth muscle tone and its reactivity and platelet aggregation were the principal physiological functions of interest. The role of dietary supplementation of omega-3 or omega-6 polyunsaturated fatty acids in preventing or potentiating the biological effects of ethanol will also be evaluated.

Methods Employed:

Male rats (300-350g) were exposed to ethanol vapors for 1-14 days before in vivo measurement of vascular tone and plasma catecholamine levels, and in vitro determination of platelet aggregation and PG production from platelet and vascular smooth muscle. An ethanol vaporization and metering apparatus with a feedback control system was previously designed and built in our laboratory. Animals were exposed to ethanol vapors for 1, 7 or 14 days and (i) lipids were extracted from platelet, aorta, heart, brain and liver and transmethylated for determination of fatty acid profiles, (ii) platelet rich plasma, aortic rings, cerebral microvasculature and brain parenchyma were prepared for in vitro PG measurement by RIA (validated by gas chromatography/mass spectrometry), (iii) platelet rich plasma was prepared for in vitro determination of platelet aggregation or (iv) the tail arteries were cannulated and connected through a harness to a tether and swivel system 24 hours prior to an assessment of blood pressure, vasoreactivity, and plasma catecholamine levels by liquid chromatography with electrochemical detection. Similar measurements were made in rats fed a diet rich in w-3 or w-6 fatty acids such as alpha or gamma-linolenic acid 5 weeks prior to and throughout the ethanol exposure period.

Major Findings:

Acute exposure (1 day) to moderate BEC resulted in a 15% elevation in mean arterial blood pressure (MABP). Plasma NE levels increased 291% in these rats and a comparable increase in plasma epinephrine levels was observed. The marked elevation in plasma catecholamines correlated strongly with elevated MABP. The pressor effect of exogenously administered NE (400ng) was lower (15%) in the ethanol exposed rat as compared to ethanol naive animals. The pressor effect of an exogenously administered TXA<sub>2</sub>-agonist was not significantly

altered. Arachidonic acid-induced platelet aggregation decreased by 37% in similarly exposed rats. This decrement correlated strongly with a 44% decrease in the capacity of these platelets to produce TXA<sub>2</sub>. However, the capacity of either an aortic ring or cerebral microvasculature preparation to produce PGI<sub>2</sub> increased 106% and 747%, respectively. Following the short-term exposure period to ethanol vapors the proportion of the eicosanoid precursor, arachidonic acid, in the total lipid extract of vascular tissue did not change.

The marked increase in plasma catecholamines may contribute to the elevation in blood pressure in acutely exposed rats whereas the associated increase in the vasodilator/platelet anti-aggregator PGI<sub>2</sub> and decrease in the vasoconstrictor/platelet aggregator TXA<sub>2</sub> may partially explain the observed decrease in vasoreactivity and platelet aggregation.

Chronic exposure (7 day) to moderate BEC did not significantly elevate MABP although plasma NE levels and plasma E levels were elevated (51% and 128%, respectively). The pressor effect of exogenously administered NE (400ng) and the TXA<sub>2</sub>-agonist (1600ng) increased (22% and 44%, respectively). Following a 14 day exposure to ethanol vapor neither MABP nor plasma catecholamines were significantly elevated, however, a vascular hyperreactivity to the pressor effect of exogenously administered NE (400ng) and the TXA<sub>2</sub>-agonist (1600ng) was observed (28% and 48%, respectively). Interestingly, at higher BEC (>180mg%) a marked vasodepression was evident. After the 7 day ethanol exposure period, ADP and arachidonic acid-induced platelet aggregation decreased by 38% and 77%, respectively. The change in arachidonic acid-induced platelet aggregation correlated strongly with a 78% decrease in the capacity of these platelets to produce TXA<sub>2</sub>. Aorta obtained from these dependent animals produced 48% less PGI<sub>2</sub> whereas the capacity of the cerebral microvasculature preparation to produce PGI<sub>2</sub> was not altered. The proportion of arachidonic acid in the total lipid extract of rat aorta decreased significantly (24%). This decrement correlated strongly with a decreased capacity to produce PG. Changes in the proportion of a fatty acid (20:4) in the total lipid extracts of platelets were comparable. Little change was observed in the fatty acyl content of the heart or brain. The capacity of the brain parenchyma to produce PG did not change with long-term ethanol exposure.

Although blood pressure was not significantly elevated following chronic (7 day) ethanol exposure, plasma catecholamines were mildly increased. The marked decrease in platelet TXA<sub>2</sub> production may partially explain decreased platelet aggregation. Moreover, the capacity to produce PG generally decreased with continued ethanol exposure and correlated strongly with as much as a 50% decrease in the proportion of its fatty acid precursor, arachidonic acid.

#### Significance to Biomedical Research and the Program of the Institute:

An increase in the PGI<sub>2</sub>/TXA<sub>2</sub> ratio may be one mechanism contributing to the lower incidence of atherosclerotic heart disease in moderate consumers of alcohol. On the other hand, mildly elevated plasma catecholamines and a marked vascular hyperreactivity to both NE and a TXA<sub>2</sub>-agonist was observed in dependent animals following a chronic ethanol exposure period. This effect may contribute to the hypertensive and vasospastic disorders found in alcoholics. Dietary

supplementation with w-6 fatty acids such as gamma-linolenic acid (18:3) may prove to be a risk factor (ie., increased platelet TXA<sub>2</sub>) or of therapeutic value (ie., increased vascular PGI<sub>2</sub>) with respect to the local role of 2-series PG in the cardiovascular system of normal and alcoholic subjects.

#### Proposed Course:

Our findings are currently being extended to include the effects of various dietary manipulations (omega 3 and 6 series fatty acids) on (i) changes in fatty acid levels, (ii) tissue (vascular, platelet, brain), and urinary eicosanoid profiles and (iii) platelet aggregation, blood pressure, pressor and depressor responses in normal and alcohol exposed subjects. The significance of altered tissue levels of docosahexaenoic acid (22:6w3) and the direct effects of 22:6 and its novel lipoxxygenated metabolites on the cardiovascular system and smooth muscle will be determined. The effect of ethanol dose and duration of exposure will be superimposed in these studies. These results will be used for formulation of carefully defined clinical protocols.

#### Publications:

Karanian, J.W., Yergey, J.L., Lister, R.L., Linnoila, M. and Salem, N. Jr.: Characterization of an automated apparatus for precise control of inhalation chamber ethanol vapor and blood ethanol concentration. Alcoholism: Clin. Exp. Res. 10:443-447, 1986.

Karanian, J.W., D'Souza, N. and Salem Jr., N.: The effect of chronic alcohol inhalation on blood pressure and the pressor response to noradrenaline and the thromboxane-mimic U46619. Life Sciences 39:1245-1255, 1986.

Karanian, J.W., Yergey, J.L. and Salem Jr., N.: The modulatory effect of prostanoids on alcohol-induced changes in the vascular state. Ann. NY Acad. Sci. 492:331-334, 1987.

Karanian, J.W. and Salem Jr., N.: Interactions of alcohol and prostaglandins in the vascular system: Implications for cardiovascular disease. In Gallo, L.L., (Ed.): Cardiovascular Disease: Molecular and Cellular Mechanisms Prevention and Treatment, Plenum Press, New York. 299-321, 1987.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00262-03 LCS
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Characterization of Oxygenated Fatty Acid Metabolites by Capillary GC/MS		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: J. Yergey Senior Staff Fellow LCS, NIAAA		
Others: N. Salem, Jr. Section Chief LCS, NIAAA H. Kim Staff Fellow LCS, NIAAA M. Linnoila Chief LCS, NIAAA A. Yoffe Chemist LCS, NIAAA		
COOPERATING UNITS (if any) Clinical Neuroscience Branch, NIMH (D. Pickard); Lab. Clinical Science, NIMH (M. Rudorfer); Neuropsychiatry Branch, St. Eliz. Hosp. (C. Kaufmann); Dept. of Neurology Services, Veterans Hosp., Wash., DC (J. Hawley); Clinical Neuropharmacology Branch, NIMH (N. Garrick)		
LAB/BRANCH Laboratory of Clinical Studies		
SECTION Section of Analytical Chemistry		
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.4	PROFESSIONAL: 1.3	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The objective of this project is to develop a sensitive and specific assay for prostaglandins in human cerebrospinal fluid (CSF) from patients with alcoholism and appropriate controls using gas chromatography / mass spectrometry. This study was initiated because of literature reports which suggested that a relationship exists between central nervous system effects of ethanol and the central production of prostaglandins. In particular, in human and animal studies, administration of prostaglandin synthesis inhibitors prior to administration of ethanol attenuated central nervous system effects of ethanol.</p> <p>Samples assayed after methodological validation was completed, contained none of the measured prostaglandins (PGE2, PGE1, PGF2a, PGF1a, 6-keto-PGF1a) at a concentration more than twice the limit of quantification (3 pg/mL CSF). Comparison of GC/MS and radioimmunoassay methods provided further validation for these results. Literature reports of much higher levels of prostaglandins in normal controls, i.e., tens to hundreds of pg/mL CSF, appear to be incorrect. Examination of monkey CSF provided a positive control, since several prostaglandins were easily quantifiable in these samples.</p>		

PROJECT DESCRIPTION:Investigators:

J. Yergey	Senior Staff Fellow	LCS, NIAAA
N. Salem, Jr.	Section Chief	LCS, NIAAA
H.Y. Kim	Staff Fellow	LCS, NIAAA
A. Yoffe	Chemist	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
D. Pickard	Section Chief	NSB, NIMH
M. Rudorfer	Staff Psychiatrist	LCS, NIMH
C. Kaufman	Medical Staff Fellow	St. Eliz. Hosp.
J. Hawley	Staff Neurologist	Veterans Hosp., Wash., D.C.
N. Garrick	Research Physiologist	CNB, NIMH

Objectives:

The specific aims of this study were: 1) To elucidate aspects of the molecular mechanisms underlying neuropathological states, with particular emphasis on alcoholism and the role of oxygenated fatty acid metabolites; 2) To develop analytical methods which can be utilized for quantification of trace amounts of oxygenated fatty acid metabolites in various biological fluids; and 3) to develop a sensitive and specific assay for prostaglandins in human cerebrospinal fluid with the goal of correlating their levels with clinical characteristics of patients.

Methods Employed:Sample assay:

Cerebrospinal fluid samples, the 22-23 mL of a 30 mL lumbar puncture, were drawn on ice into polypropylene tubes containing indomethacin to inhibit prostaglandin production. Exactly 2 mL was transferred to a second tube containing internal standards (250 pg each of tetradeuterated PGE<sub>2</sub>, PGF<sub>2a</sub>, and 6-keto-PGF<sub>1a</sub>), and was frozen at -70°C. Thawed samples were brought to pH 3.5 with formic acid and extracted using 1 mL Supelclean LC-18 cartridges as follows: samples were applied, rinsed with 2 mL of 20% methanol, dried for 5 min with nitrogen, rinsed with 2 mL of benzene, and the prostaglandins eluted in 2 mL of methanol directly into silanized glass vials for derivatization. Extracted samples were derivatized to the methoxime, pentafluorobenzyl ester, trimethylsilyl ether, and assayed by capillary gas chromatography / negative chemical ionization mass spectrometry using cold on-column injection. The PFB ester is a highly electro-negative species which is available for efficient electron capture in a mass spectrometer ion source operated in negative chemical ionization mode, providing further selectivity to the method. The mass spectrometer was operated in selected ion monitoring mode, i.e. only the masses corresponding to the major ion for each prostaglandin were monitored, thus greatly increasing the sensitivity of the technique. Quantification of the prostaglandins is made by comparison to signals for the tetradeuterated standards added to the sample.

Radioimmunoassay (RIA) - GC/MS comparison:

CSF samples which had been stored without prior addition of tetradeuterated internal standards were extracted and assayed by both methods for PGE<sub>2</sub> and 6-keto-PGF<sub>1a</sub>. Duplicate CSF samples, collected at the same time, were spiked with 13 pg/mL of both prostaglandins, extracted, and again assayed by both methods.

Monkey CSF:

In order to investigate another primate source of CSF, but with the advantage of larger sample sizes and multiple samplings, Rhesus monkey samples were drawn and assayed with the same method as human samples.

Major Findings:Patient CSF:

Levels of the primary prostaglandins (PGE<sub>2</sub>, PGE<sub>1</sub>, PGF<sub>1a</sub>, PGF<sub>2a</sub>, and 6-keto-PGF<sub>1a</sub>) in human cerebrospinal fluid from normal controls and alcoholics following 3 weeks abstinence were at or below the limit of quantification (3 pg/mL CSF). Internal standards were recovered in good yield, (> 75%), indicating that sample storage, extraction, and derivatization were proceeding reliably. These data suggest that literature reports of much higher levels in normal controls, i.e. tens to hundreds of pg/mL CSF, are incorrect.

Radioimmunoassay (RIA) - GC/MS comparison:

Unspiked CSF samples contained no quantifiable PGE<sub>2</sub> or 6-keto-PGF<sub>1a</sub> by either method. Both methods were able to detect the 13 pg/mL of each prostaglandin spiked into the duplicate samples with acceptable precision and accuracy. Both methods can, therefore, be used reliably for the measurement of low concentrations of prostaglandins.

Monkey CSF:

At the present time only samples which were collected while the monkeys were recovering from anesthesia were available. Several of the assayed prostaglandins PGE<sub>2</sub>, PGF<sub>2a</sub>, 6-keto-PGF<sub>1a</sub> were present in each of the monkey samples and were easily quantifiable. These samples provided a positive control, indicating that endogenously produced prostaglandins were indeed quantifiable using our method.

Significance to Biomedical Research and the Program of the Institute:

These investigations have validated the capability for sensitive and selective assay of prostaglandins. Furthermore, it has been shown that concentrations of primary prostaglandins are below the 3 pg/mL level in normal human CSF and in CSF from alcoholics following three weeks abstinence, in contrast to literature reports of much higher levels.

Proposed Course:

The assay for primary prostaglandins in human cerebrospinal fluid will be applied to samples collected from alcoholics during acute withdrawal where elevated levels might be expected, as well as other patient types. Further investigations of monkey CSF samples will also be pursued in order to possibly elucidate the reasons for the extremely low levels of prostaglandins observed in normal human CSF. The methodologies will also be expanded to include other prostanoids, leukotrienes, and metabolites of other fatty acids.

Publications:

None



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00244-04 LCS
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Ethanol-induced Changes in B-Endorphin and CRF Binding to Peripheral Tissue		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	R. Eskay	Section Chief  LCS, NIAAA
Others:	J. Dave	Visiting Associate  LCS, NIAAA
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Clinical Studies		
SECTION Section of Neurochemistry		
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.5	PROFESSIONAL: 1.3	OTHER: 0.2
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>An understanding of the effect of ethanol on the hypothalamic-pituitary-adrenal axis (HPAA) requires an appreciation of the central and peripheral regulatory components which constitute the HPAA. Intimately involved in the regulation of the HPAA are hypothalamic- and possibly peripheral-derived corticotropin-releasing hormone (CRF) and pituitary-gland derived beta-endorphin (BE), which is co-secreted with ACTH. Specific binding sites for CRF and BE have been identified in various rat peripheral tissues and bovine chromaffin cells in culture. Occupancy of the CRF binding sites in rat adrenal membranes and bovine chromaffin cells or BE binding sites in hepatic membranes activate the adenylate cyclase/cAMP system. Furthermore, peripheral-tissue binding sites for CRF and BE appear to be modulated by glucocorticoids as do pituitary CRF binding sites.</p> <p>Follow-up experiments, in which rats were exposed to ethanol vapors for 14 days revealed reduced pituitary CRF binding and reduced CRF binding to a variety of CRF binding sites, including what was initially demonstrated to be reduced CRF erythrocyte (RBC) binding. These observations suggested that CRF binding to presumed RBC membranes and pituitary membranes may be modulated in a similar direction. Furthermore, it was reasoned that establishment of this concept would be an important contribution in the area of clinical neuroendocrinology.</p> <p>This project has been terminated.</p>		



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b> Z01 AA 00252-04 LCS
<b>PERIOD COVERED</b> October 1, 1986 to September 30, 1987		
<b>TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)</b> <u>The Effect of Ethanol on POMC Peptide Synthesis and Release In Vivo and In Vitro</u>		
<b>PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)</b>		
<b>PI:</b> R. Eskay	Section Chief	LCS, NIAAA
<b>Others:</b> K. Mishler	Microbiologist	LCS, NIAAA
J. Dave	Visiting Associate	LCS, NIAAA
A. Thiagarajan	Visiting Fellow	LCS, NIAAA
W. Chen	Senior Staff Fellow	LCS, NIAAA
<b>COOPERATING UNITS (if any)</b>  None		
<b>LAB/BRANCH</b> Laboratory of Clinical Studies		
<b>SECTION</b> Section of Neurochemistry		
<b>INSTITUTE AND LOCATION</b> NIAAA, 9000 Rockville Pike, Bethesda, MD 20892		
<b>TOTAL MAN-YEARS:</b> 2.0	<b>PROFESSIONAL:</b> 1.5	<b>OTHER:</b> 0.5
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)</b>  Endogenous neuropeptides constitute a growing list of biologically important molecules with both central and peripheral sites of action and loci of synthesis. In spite of the obvious importance of biologically-active peptides in regulating both endocrine and neural events, lacking is a detailed evaluation of the effects of ethanol on even a single neuropeptide system. Since the administration of ethanol is known to alter certain regulatory aspects of the hypothalamic-pituitary-adrenal axis (HPAA) and since the integrity of this system depends on the synthesis and secretion of specific regulatory neuropeptides at the hypothalamic (e.g., corticotropin-releasing hormone (CRF); vasopressin) and pituitary gland (e.g., beta endorphin (BE); ACTH) level, we have been evaluating the impact of ethanol on the HPAA as a model system in vivo and in vitro. Realizing the necessity of understanding the direct versus indirect effects of ethanol at the cellular level, we have been working with AtT-20 cells, corticotropes, and melanotropes in culture and ethanol-exposed intact male rats. It has been possible to study the effects of ethanol on such primary signal transduction systems as the adenylate cyclase (AC)/cyclic (c) AMP system and the phosphoinositide protein kinase C system. It is envisioned that the exploration of each of the component parts of the HPAA (i.e., CNS, pituitary and adrenal gland) independent of one another and the subsequent intergration of results from the intact animal will enable one to profile the acute, chronic, direct and indirect effects of ethanol at the cellular and subcellular level, as well as the primary versus secondary sites of ethanol-induced perturbations of the HPAA. Since neuroendocrine cells are continuously synthesizing and secreting products, our studies in their broadest sense are designed to understand the effects of ethanol on stimulus-secretion-synthesis coupling in secretory cells. To date our investigations have yielded a variety of information.		

PROJECT DESCRIPTION:Investigators:

R. Eskay	Section Chief	LCS, NIAAA
K. Mishler	Microbiologist	LCS, NIAAA
J. Dave	Visiting Associate	LCS, NIAAA
W. Chen	Senior Staff Fellow	LCS, NIAAA
A. Thiagargan	Visiting Fellow	LCS, NIAAA

Objectives:

The ongoing aims of this group of experiments are: 1) to study the biosynthesis and regulation of release of neuropeptides from the pituitary gland, CNS and tumor cell lines, which includes an understanding of the sequence of events from membrane-receptor activation to intracellular-messenger systems to physiological responses (neuropeptide release) and 2) to determine the concentration-dependent and time-dependent effects of ethanol on the various cellular events as outlined in 1, both in vivo and in vitro.

Methods Employed:

AtT-20 cells, an anterior pituitary derived tumor cell line, and dispersed anterior pituitary (AP) and intermediate lobe (IL) cells, which synthesize and secrete POMC-neuropeptides, were cultured. Prior to the one hour incubation of cells with various secretagogues with or without ethanol, the culture medium was removed and the cells were preincubated for up to 24 hours in the presence of ethanol. Following the one-hour experimental incubation the medium was removed and centrifuged to remove detached cells. The supernatant fluid was obtained for the determination of beta-endorphin and cyclic AMP by radioimmunoassay. In certain experiments the binding of [ $I^{125}$ ]-corticotropin-releasing hormone to and POMC messenger RNA (mRNA) in cultured cells was evaluated.

Major Findings:Effect of acute or chronic ethanol exposure on the HPA axis in vivo

Animals exposed continuously to ethanol vapors for up to 14 days in an inhalation chamber with continuous blood ethanol levels between 100-250 mg% were found to have 25-30% lower CRF binding capacity and basal AC activity in membranes from endocrine tissue (e.g. AP, IL and adrenal membranes) and non-endocrine peripheral tissue (i.e., liver and kidney). In addition chronic ethanol treatment rendered CRF-responsive tissue, as determined by monitoring membrane-associated adenylate cyclase activity, refractory to subsequent CRF stimulation. Chronic ethanol exposure resulted also in a time-related decrease in POMC mRNA levels in both the AL and IL of the pituitary gland. Paralleling the apparent reduction of POMC mRNA levels was a concomitant reduction of plasma BE levels which is one of several stress related peptides derived from the POMC precursor molecule. Of particular interest is the finding that IL lobe POMC mRNA levels, which reflect the biosynthetic activity of cells expressing this message, are more sensitive to ethanol exposure than AP-POMC mRNA levels. This result would argue that ethanol's effects, at least on IL cells, are direct and not influenced by elevated glucocorticoid levels, since elevated glucocorticoids do not inhibit POMC peptide synthesis or secretion from IL cells.

In order to explore certain of the apparent contradictions between the acute and chronic effects of ethanol on the HPAA, adult male rats received a single infusion of ethanol via a chronic indwelling intragastric catheter followed by the determination of plasma ACTH, corticosterone (CS) and catecholamines (epinephrine (EPI), norepinephrine (NOREPI), and dopamine (DA)). Blood ethanol concentrations determined at 15,30,60, 90 and 150 min. post-ethanol infusion were similar at all sample times and ranged from 240-280 mg%. Following ethanol infusion, plasma EPI levels increased 6-10 fold at 15 min. and returned to basal levels by 60 min. NOREPI levels increased approximately 2 fold by 15 min. post-ethanol infusion and remained elevated for up to 2 hrs., whereas, DA levels remained unchanged. Basal plasma ACTH and CS levels exhibited a diurnal rhythm and ethanol-induced increases in ACTH and CS release over basal, as determined 1 hr post ethanol infusion, were maximal when basal ACTH and CS were at their nadir. The maximal observed increases in ACTH and CS levels were 10- and 4-fold respectively. Consistent with the knowledge that stress hormone secretion (ACTH, BE) is under multifactorial control, it would appear that activation of the HPAA and subsequent secretion of ACTH via a single dose of ethanol is also. If one administers vasopressin antiserum or removes the source of plasma EPI through adrenal demedullation, plasma ACTH levels are significantly blunted following a single-dose ethanol challenge.

#### Effect of acute or chronic ethanol treatment on cultured AtT-20 and pituitary cells in vitro.

Incubation of AtT-20 or AP cells in the presence of ethanol (0.1-0.4%) for less than 24 hrs has only a marginal effect on basal or secretagogue-induced BE release and cAMP levels; however, incubation of both cell types in the presence of 0.2-0.4% ethanol for 24 hrs. or longer results in a reduction of basal and secretagogue (CRF, forskolin, isoproterenol, 8-bromo-cAMP, dibutyl-cAMP)-induced cAMP levels and BE secretion. In contrast to the observed ethanol-induced inhibition of the AC/cAMP-mediated secretagogue response, the ability of the calcium ionophores ionomycin or A23187 or 50mM  $K^+$  to enhance BE secretion from AP cells was not altered by preincubation in the presence of ethanol. It would appear that chronic ethanol treatment alters AC/cAMP mediated BE secretion, and that enhanced intracellular  $Ca^{++}$  levels can override ethanol's inhibition of BE secretion. Since all of the secretagogues used to enhance BE secretion in this study either increase cAMP levels or mimic increased cAMP levels and enhance cytosolic  $Ca^{++}$  pools, the primary ethanol-induced dysfunction may be a blockade of  $Ca^{++}$  entry through either voltage-dependent  $Ca^{++}$  channels or other membrane  $Ca^{++}$  gating mechanisms.

In addition to observed effects of ethanol on BE secretion, ethanol produced a dose- and time-dependent decrease in POMC mRNA levels in AtT-20, AP and IL cells. 24 hr. treatment of AtT-20 cells with 0.2, 0.4 or 0.6% ethanol produced approximately 0, 60 or 80% decrease in POMC mRNA levels, respectively. Treatment of AtT-20 cells with 0.4% ethanol for 8, 12 or 24 hrs. produced a 40, 50 or 60% decrease in POMC mRNA levels, respectively. Similarly, treatment of AP or IL cells with 0.2, 0.4 or 0.6% ethanol for 24 hrs. produced a 40, 60, or 60% decrease in POMC mRNA levels, respectively. Although ethanol directly inhibits POMC mRNA synthesis in each cell type studied, this inhibition of synthesis is not indiscriminate and was not altered in lactotrophs in either in vitro or in vivo studies.

Finally, we have monitored the binding characteristics of [ $^{125}I$ ]-ovine CRF to membrane fractions derived from AtT-20 cells following 24 hrs. ethanol (0.4%) pretreatment. In five independent experiments neither the dissociation

constant ( $K_d$ ,  $2.6 \pm 0.6 \text{ nM}$ ) nor the receptor concentration ( $B_{\text{max}}$ , 70-80 fmoles/mg protein) varied with ethanol treatment. This suggests that ethanol's ability to inhibit CRF-induced hormone secretion in vitro lies beyond the level of membrane-receptor activation.

#### Significance to Biomedical Research and the Program of the Institute:

The continued exploration of the effects of ethanol on fundamental cellular events, utilizing well-characterized in vivo and in vitro model systems should provide an understanding of the multiple, ethanol-induced perturbations of normal cellular functions. This, in turn, will hasten the development of effective therapeutic agents to treat patients with alcohol-related dysfunctions.

#### Proposed Course:

These projects are continuing with special emphasis on delineating the effects of ethanol on secretagogue-induced interacellular  $\text{Ca}^{++}$  redistribution and/or  $\text{Ca}^{++}$  entry from the extracellular environment. The possibility that ethanol alters the redistribution or synthesis of a specific phosphorylated protein which is involved in mediating stimulus-secretion-synthesis coupling in AtT-20 cells will also be evaluated.

#### Publications:

Bisserbe, J.C., Patel, J., and Eskay, R.L.: Evidence that the peripheral-type benzodiazepine receptor ligand Ro 5-4864 inhibits beta endorphin release from AtT-20 cells by blockade of voltage dependent calcium channels. J. Neurochem. 47:1419-1424, 1986.

Dave, J.R., Eiden, L.E., and Eskay, R.L.: Differential effects of CRF, forskolin and phorbol ester on B-endorphin release and pro-opiomelanocortin synthesis in cultured anterior pituitary and AtT-20 cells. Ann. N.Y. Acad. Sci. 493:577-580.

Dave, J.R., and Eskay, R.L.: Ethanol decreases corticotropin-releasing factor binding adenylate cyclase activity, pro-opiomelanocortin biosynthesis, and beta-endorphin release in cultured pituitary cells. Ann. N.Y. Acad. Sci. 492: 327-330, 1987.

Ishac, E., Eskay, R., Hirata, F., Axelrod, J. and Kunos, G.: Adrenergic regulation of beta-endorphin secretion from anterior-pituitary in conscious rats: Effects of thyroid state. Endocrinology 120:1073-1078, 1987.

Dave, J., Eiden, L., Lozousky, D., Waschek, J., and Eskay, R. Calcium-independent and calcium-dependent mechanisms regulate corticotropin-releasing factor stimulated proopiomelanocortin peptide secretion and messenger ribonucleic acid production. Endocrinology 120:305-310, 1987.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00253-03 LCS												
PERIOD COVERED October 1, 1986 to September 30, 1987														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Characterization and Regulation of Release of Atrial Natriuretic Peptides														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: R. Eskay</td> <td style="width: 33%;">Section Chief</td> <td style="width: 33%;">LCS, NIAAA</td> </tr> <tr> <td>Others: J. Dave</td> <td>Visiting Associate</td> <td>LCS, NIAAA</td> </tr> <tr> <td>K. Mishler</td> <td>Microbiologist</td> <td>LCS, NIAAA</td> </tr> <tr> <td>F. Hoffer</td> <td>Microbiologist</td> <td>LCS, NIAAA</td> </tr> </table>			PI: R. Eskay	Section Chief	LCS, NIAAA	Others: J. Dave	Visiting Associate	LCS, NIAAA	K. Mishler	Microbiologist	LCS, NIAAA	F. Hoffer	Microbiologist	LCS, NIAAA
PI: R. Eskay	Section Chief	LCS, NIAAA												
Others: J. Dave	Visiting Associate	LCS, NIAAA												
K. Mishler	Microbiologist	LCS, NIAAA												
F. Hoffer	Microbiologist	LCS, NIAAA												
COOPERATING UNITS (if any)  None														
LAB/BRANCH Laboratory of Clinical Studies														
SECTION Section of Neurochemistry														
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892														
TOTAL MAN-YEARS: 2.00	PROFESSIONAL: 1.75	OTHER: .25												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Cardiac atria contain a family of peptides, collectively termed atrial natriuretic peptides (ANPs), which are derived from a common precursor and possess intrinsic natriuretic, diuretic, vasorelaxant and endocrine modulatory effects. Furthermore, recent immunocytochemical and radioimmunoassay (RIA) distribution studies have localized ANPs to areas of the CNS that suggest the ANPs are involved in central cardiovascular regulatory events and in the release and/or synthesis of certain pituitary gland hormones.</p> <p>Findings revealed the presence of at least two plasma forms of ANP ranging from 24-28 amino acids. Additional studies revealed that volume loading or pressor agents (e.g. adrenaline, vasopressin) which cause atrial stretch result in a rapid increase in circulating ANPs. The acute administration of ethanol to rats in order to achieve blood ethanol levels in the range of .2-.25% did not alter plasma levels of ANPs or vasopressin; however, chronic exposure of rats to ethanol vapors for 7-14 days lowered plasma ANP levels. On the basis of results obtained in vitro from cultured adult rat atrial cardiocytes, it would appear that our observed enhancement of ANPs in vivo are indirectly mediated since a number of pressor agents did not alter ANP secretion in vitro. To date only drugs or experimental conditions which alter intracellular Ca++ levels appear to modulate ANP secretion in cultured atrial myocytes.</p> <p>This project has been terminated.</p>														

PROJECT DESCRIPTION:Investigators:

R. Eskay	Section Chief	LCS, NIAAA
J. Dave	Visiting Associate	LCS, NIAAA
K. Mishler	Microbiologist	LCS, NIAAA

Objectives:

The specific aims of these studies are to 1) understand the biosynthesis and secretion of ANPs from the cardiac atria and 2) to determine if ethanol-induced alterations in circulating ANPs or tissue responsiveness to ANPs are responsible in part for certain cardiovascular system pathologies associated with alcoholism, such as hypertension and edema.

Methods employed:

Plasma levels of ANPs were determined by RIA following established protocols for the extraction of peptides from plasma. Blood samples were obtained from conscious rats with indwelling arterial catheters following volume loading with either physiological saline, 5% glucose or the administration of pressor agents or ethanol. For the characterization of plasma ANPs, plasma extracts or synthetic ANPs were subjected to HPLC utilizing a linear gradient of acetonitrile (ACN) from 25%-55% ACN (Pump A, 0.1% TFA in water; Pump B, 0.1% TFA in ACN) one ml fractions were collected over 60 min, dried down and assayed. Established tissue culture methods were used in in vitro cardiocyte experiments.

Major findings:

In conscious rats with chronic indwelling catheters, volume loading with isotonic saline or glucose enhanced circulating immunoreactive ANPs approximately four-fold and the circulating, physiologically important ANPs in the rodent appear to be alpha-rat ANP<sub>5-28</sub> (atrioepetin III) and alpha-rat ANP<sub>1-28</sub> (ANF). Furthermore, ug amounts of adrenaline or carbachol and ng amounts of vasopressin administered as a bolus-arterial injection resulted in a two- to five-fold increase in circulating ANPs within minutes. Plasma levels of ANPs remained unchanged following the acute administration of ethanol, whereas chronic ethanol treatment lowered plasma ANPs. Finally, agents which result in the redistribution of intracellular Ca<sup>++</sup> or increase Ca<sup>++</sup> flux across the plasma membrane result in enhanced secretion of ANP's from cultured cardiocytes in vitro.



Significance to Biomedical Research and the Program of the Institute:

Alteration of the purported role for ANPs in disorders of electrolyte balance or blood-pressure regulation could be a factor in the development of alcohol-related cardiovascular disease. Since ethanol is known to alter vascular tonus and the ANPs are known to antagonize the pressor actions of certain substances (e.g. noradrenaline and angiotensin II), the possible effects of ethanol on ANP release or ANP sites of actions (e.g. kidney, vascular smooth muscle, and pituitary gland) are worth exploring.

Proposed course:

These studies as a separate entity were terminated in January 1987.

Publications:

Samson, W.K., and Eskay, R.L.: Endocrine and neuroendocrine actions of cardiac peptides. In Moody, T.(Ed.): Neural and Endocrine Peptides and Receptors. New York, Plenum (1986).

Palkovits, M. and Eskay, R.: Atrial natriuretic peptide in the median eminence is of paraventricular nucleus origin. Neuroendocrinology (in press).



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00254-03 LCS
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) PKC and the Secretion and Biosynthesis of Neuropeptides in AtT-20 Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	R. Eskay	Section Chief LCS, NIAAA
Others:	R. Parthasarathy K. Mishler	Visiting Associate Microbiologist LCS, NIAAA LCS, NIAAA
COOPERATING UNITS (if any) Biological Psychiatry Branch, NIMH (J. Patel)		
LAB/BRANCH Laboratory of Clinical Studies		
SECTION Section of Neurochemistry		
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Enhanced protein phosphorylation is one of the intracellular events which invariably follows membrane receptor activation. Recently identified Protein Kinase C (PKC) is a unique enzyme which phosphorylates specific proteins, whose functions remain to be determined. PKC requires a phospholipid and a diacylglycerol (DAG) for maximal activity. DAG is the hydrolytic product of membrane polyphosphoinositide (PI) breakdown, which occurs following membrane receptor occupancy, and is one of the initial events in signal transduction. The release of DAG during PI turnover substantially stimulates PKC activity over that observed in quiescent cells. The known co-carcinogen or tumor promoter, 12-O-tetradecanoylphorbol 13-acetate (TPA) specifically increases membrane PKC activity. Associated with this increased membrane PKC activity is a reduction of cytosolic PKC activity, which suggests that translocation of PKC from the cytosol to the membrane is an important intracellular event.</p> <p>TPA treatment of AtT-20 cells resulted in a dose-related increase in BE secretion and translocation of PKC from the cytosolic to the membrane fraction. A maximally-stimulating concentration of TPA enhanced membrane PKC activity 2-fold within 1 min and 5-fold within 3 min. Preincubation of AtT-20 cells with ethanol (0.1-0.6%) for 24 hours resulted in a dose-related 2-5 fold reduction in cytosolic PKC activity and a 50% reduction in membrane PKC activity at 0.4% ethanol. Ethanol pretreatment for 24 hours only marginally reduced the ability of a maximal stimulatory concentration of TPA to induce translocation of PKC.</p> <p>This project has been terminated.</p>		



**ANNUAL REPORT OF THE LABORATORY OF METABOLISM  
AND MOLECULAR BIOLOGY  
NATIONAL INSTITUTE ON ALCOHOL ABUSE AND ALCOHOLISM  
October 1, 1986 to September 30, 1987  
Richard L. Veech, M.D., D.Phil., Acting Chief**

**Introduction**

**Programs of the Laboratory of Metabolism and Molecular Biology**

The programs of the laboratory bring a diversity of powerful biochemical disciplines of metabolic control, molecular genetics and physical biochemistry to bear on the problems of alcoholism and its associated pathology. During the year, the laboratory was divided into three sections which reflect the laboratory programs and which are:

1. Section on Molecular Genetics
2. Section on Metabolic Control
3. Section on Physical Chemistry

**Changes in Personnel**

During the past year, several distinguished senior scientist have joined the laboratory. Dr. Janet Passonneau, an acknowledged leader in the field of microanalytical neurochemistry, has joined the staff of the laboratory in the Section on Metabolic Control. Dr. Alan McLaughlin, a leader in the field of nuclear magnetic resonance, moved to the laboratory with several of his staff from the University of Pennsylvania to head a new initiative in NMR, both of metabolic processes and of the structure of cell membranes. Dr. Joseph Casazza, whose work defined for the first time the metabolic pathway leading to the elevation of serum 1,2-propandiol in a significant proportion of human alcoholics, was appointed acting head of the Section on Molecular Genetics. Working with Dr. Casazza on the genetic aspects of alcoholism, Dr. B.J. Song, who made a significant advance in the field of alcohol studies by cloning of the specific  $P_{450}$  IIE induced in some people by the excessive consumption of alcohol, is continuing his work of cloning of gene products which are candidates as markers for the predisposition to develop alcoholism or the physical toxicities resulting from alcohol abuse.

Dr. Harish Pant, who was transferred into the Laboratory in May 1986, left the laboratory in June 1987, to accept a position in the National Institute of Child Health.

**Summary of the Work of the Three Sections of the Laboratory**

**1. Section on Molecular Genetics**

The projects carried out by this section fall into several main categories:

- A. The mechanism of enzymatic changes induced by ethanol consumption;
- B. The identification of genetic markers for the predisposition to alcoholism;
- C. The effects of ethanol upon the action of cellular growth factors.

**A. The Mechanisms of Enzymatic Changes Induced by Ethanol Consumption**

For at least twenty years, a subject of major interest in alcohol research had been the hypertrophy of microsomes which is observed to occur following alcohol consumption (Lieber C.S., DeCarli L.M. J Biol Chem 245: 2505-2512, 1970). Over the past four years, the workers in this laboratory have:

1. defined the major metabolic pathway of microsomal metabolism induced by the consumption of ethanol and how that pathway interacts with the normal cellular metabolic pathways (Casazza, J.P., Felver M.F., Veech R.L.: J Biol Chem, 259: 231-236, 1984);
2. identified the specific microsomal enzyme induced by ethanol consumption (Koop, D.R., Casazza, J.P.: J Biol Chem 260: 13607-13612, 1985; and
3. cloned the microsomal enzyme induced by ethanol ingestion (Song, B.J., Gelboin, H.V., Park, S.S., Yang, C.S., Gonzales, F.J.: J Biol Chem 261: 16689-16697, 1986).

The practical sign of the induction of this enzyme is the finding of elevated levels of 1,2-propanediol in the serum of a majority of alcoholic patients as well as a significant number of non-alcoholics with a history of social drinking within currently accepted normal limits (Casazza, J.P., Morgan, M.Y., Veech, R.L.: unpublished observations).

Consumption of ethanol also results in the induction of delta aminolevulinic acid synthetase, the mitochondrial enzyme catalyzing the first, and in liver the rate controlling step of porphyrin and heme biosynthesis. The enzyme from mouse liver was successfully cloned this year (Schoenhaut, D.S., Curtis, P.J.: Gene 48: 55-63, 1986) and shows extensive homology with the chicken liver enzyme. Studies in this laboratory in isolated rat hepatocytes are continuing aimed at: (1) determining the mechanism whereby ethanol increases the rate of enzyme synthesis and (2) the mechanism whereby a higher molecular weight precursor of the enzyme, which is synthesized in the cytoplasm is transported into the mitochondrial matrix.

#### B. The Identification of Genetic Markers for Alcoholism

While there is a genetic factor which predisposes to the development of alcohol addiction, the nature of the gene or genes responsible for this predisposition are not known. (See Rutstein, D.D., Veech, R.L.; Genetics and Alcoholism, Epidemiological Aspects; and Veech, R.L., Gitomer, W.L., Casazza, J.P., Metabolic pathways leading to diol production. In: Goedde H.W., Agarwal, D.P. (Eds.): Genetics and Alcoholism. New York, Alan R. Liss, 1987, pp. 33-46; 185-200). A promising lead in this search for a genetic marker for alcoholism is the reports of increased levels of d or l-2,3-butanediol in the presence of the serum of alcoholic patients consuming distilled spirits. The most comprehensive study of the specificity of the presence of d or 2,3-butanediol yet undertaken was completed this year on over 250 subject referred to the Royal Free Hospital, London, with all forms of biopsy proven liver disease. In this study, it was found that over 25% of patients with alcoholic cirrhosis, even when abstinent from alcohol by history and measurement of ethanol, showed elevation of serum d or 2,3-butanediol.

Studies are continuing to determine the enzymatic pathway responsible for the production of this unusual metabolite in alcoholic and cloning of enzymes likely to be responsible for its production is now well underway. Should the enzymes currently being cloned in fact prove to be responsible for the production of d or 2,3-butanediol in humans, this should allow for prospective screening of patients at high risk for the development of alcohol addiction.

#### C. The Effects of Ethanol Upon Actions of Cellular Growth Factors

An important effect of ethanol is to disrupt cellular growth. In its most dramatic form, these effects are seen in the facial abnormalities and brain malformations seen in the fetal alcohol syndrome which is now being reported as one of the leading causes of mental retardation in the United States. It is also less obviously apparent in the disordered regrowth of hepatic cells which is characteristic of alcoholic hepatitis and cirrhosis. Work completed in the laboratory this year

has identified for the first time the sites of intracellular action of two important growth regulating products of oncogenes, epidermal growth factor and platelet derived growth factor.

For the first time ever reported in the living animal, it was found that platelet derived growth factor increased the  $V_{\max}$  of glucose-6-phosphate dehydrogenase with changing the  $K_m$  of the enzyme for its various substrates (Reed, B.Y., King, M.T., Gitomer, W.L., Veech, R.L.: J Biol Chem 262: 8712-8715, 1987). Studies are now underway to determine the mechanism of this change.

In a second series of experiments, it was found that epidermal growth factor changed the kinetic parameters of the enzyme pyruvate kinase with resultant changes in the metabolite levels at the beginning and the end of the glycolytic pathway. The degree of phosphorylation of glucose to product glucose 6-P was changed as was the degree of phosphorylation of PEP to pyruvate. Interestingly the changes induced around the metabolites of pyruvate kinase were abolished by ethanol administration. This finding is of potential interest in understanding the mechanism of the fetal alcohol syndrome, since epidermal growth factor is of particular significance in control the growth and development of facial structures, which are the cardinal diagnostic sign found in the fetal alcohol syndrome. Work to determine the mechanisms of these acute changes at these two sites of epidermal growth factor are now well underway.

Finally, the role of thyroid hormone and its blockade by the antithyroid agent, propylthiouracil, has been the subject of continuing interest as a result of the clinical trails of this drug in the treatment of alcoholic hepatitis by Israel and his co-workers at the Addiction Research Foundation in Toronto. The interaction between thyroid hormone and growth hormone on the induction of enzyme activities in liver were the subject of study published from the laboratory during the past year (Reed, B.Y., Veech, R.L.: BBRC 141: 78-83, 1986.)

## 2. Section on Metabolic Control

The programs of this section are centered in two major areas:

- A. the interaction between ethanol and its metabolites and inorganic ion distribution;
- B. the uses of  $^{31}\text{P}$  NMR spectroscopy in the study of metabolic diseases;
- C. the development of isotopic techniques for the measurement of metabolic rates.

### A. The Interaction Between Ethanol and its Metabolites and Inorganic Ion Distribution.

The major metabolic product of ethanol is acetate. It has been found that the metabolism of acetate by liver leads to profound redistribution of cellular calcium. The accumulation of calcium by liver during acetate metabolism can result in an increase in liver calcium by over 4 fold in ten minutes due to precipitation within the mitochondrial matrix of insoluble calcium pyrophosphate during the activation of the short chain fatty acids (Gitomer, W.L., Veech, R.L.: Toxicology and Industrial Health 2: 299-307, 1986).

It follows from these easily observable events, that the free mitochondrial  $[\text{Ca}^{2+}]$  is likely to approximate the concentration of free  $[\text{Ca}^{2+}]$  present in serum indicating that a very large gradient of free  $[\text{Ca}^{2+}]$  exists across the inner mitochondrial membrane in vivo. This finding has implications for the fundamental regulation of mitochondrial ATP production.

It has been postulated that there is a definable relationship between the distribution of common cellular electrolytes and cellular water and the cellular phosphorylation potential (Veech, R.L.: Pyridine Nucleotide Coenzymes: Chemical, Biochemical, and Medical Aspects, Vol 2B: Dolphin, D., Poulson, R., Avramovic, O. (Eds): New York, John Wiley & Son, 1987, pp. 79-104). One of the best known effects of ethanol is to cause a reduction of the free cytosolic  $[\text{NAD}^+]/[\text{NADH}]$

ratio and with that, a decrease in the free cytosolic  $[ATP]/[ADP][P_i]$  ratio. Such a change would result in a redistribution of the cellular electrolytes,  $Na^+$ ,  $K^+$ ,  $Cl^-$ ,  $Ca^{2+}$  and water. The relationship of these changes in cellular water to the pathological changes associated with liver damage and to the mechanisms whereby ethanol produces anesthesia are being investigated.

### B. The Uses of Nuclear Magnetic Resonance in Metabolic Resonance

Nuclear magnetic resonance (NMR) is an increasingly important tool both in imaging and spectroscopy. The physical chemical basis for the interpretation of the major signals found in tissue with  $^{31}P$  NMR stems from earlier work of this laboratory on the relationship between ratio of  $[ATP]/[ADP]$  and the ratio of  $[creatine]/[creatine\ P]$   $[H^+]$  ratio (Veech, R.L. et al: J Biol Chem 254: 6538-6547, 1979). The effects of hypercapnea and the resultant acidosis on brain NMR signals, as occurs after the ingestion of significant quantities of ethanol have been investigated (Nioka, S., Chance, B., Hilberman, M., Subramanian, H.V., Leigh, J.S., Veech, R.L., Foster, R.E.: J Appl Physiol 62: 2094-2102, 1987).

Studies on the effects of acetate, the primary metabolite of ethanol, upon the perfused rat heart using  $^{31}P$  NMR and conventional metabolite measurements, coupled with estimates of efficiency of work and  $O_2$  consumption are currently underway in collaboration with workers at the University of Pennsylvania where a 5 Tesla magnet is available giving high resolution spectra.

### C. The Development of Isotopic Techniques for the Measurement of Metabolic Rate.

Over the past decade, a great deal of interest has developed over the use of flurodeoxyglucose as a proposed agent for use in the quantitative measurement of regional cerebral metabolic rates. Work from this laboratory has indicated that such a method is only semiquantitative at best. A long standing controversy about the rate of the dephosphorylation of glucose 6-P in brain was finally concluded this year with the publication of a letter to Science presenting data which we believe shows that the rate of this reaction is about 20 to 25% of the rate of glucose phosphorylation (Huang, M.-T., Veech, R.L.: Glucose-6-Phosphatase Activity in Brain. Science 234: 1128-1129, 1986.) A rebuttal to this paper by Nelson, T., Dienel, G. and Sokoloff, L., follows the article from this laboratory. It is our present opinion that the rebuttal does not vitiate the points made, and further work on this project is terminated.

### 3. Section on Physical Chemistry

A new section on physical chemistry was created this year, headed by Dr. Alan McLaughlin who joined the laboratory from the University of Pennsylvania, where he had formerly worked with Dr. Britton Chance, Dr. Mildred Cohen and Dr. Jack Leigh. As is suggested by his former colleagues, Dr. McLaughlin is an expert in the techniques of NMR and its applications to biological systems. With Dr. McLaughlin, a staff of five new laboratory members have joined this section to make use of new NMR equipment housed in the new NMR facility on the NIH main campus. The new equipment and facilities are expected to be operational by the fall of 1987. At this time, three major projects are underway:

- A. NMR measurement of organ blood flow;
- B. NMR studies of pancreatic metabolism;
- C. Studies of relationship between membrane surface charge density and surface potential.



#### A. NMR Determination of Organ Blood Flow

The blood flow to particular organs (e.g., the brain) can vary among individual animals and can change in response to physiological perturbations. In order to interpret metabolic information on individual animals, and to compare metabolic information from different animals, it is important to measure organ blood flow at the same time NMR data is obtained.

The standard experimental techniques for the determination of organ blood flow in animals are either invasive or cannot be used in the NMR magnet. In the last year, we have used an NMR technique, which is an adaptation of the Kety-Schmidt approach, to monitor blood flow in the brains of experimental animals. We have tested this new NMR approach using the radioactive microsphere approach. We found that the NMR determinations of cortical cerebral blood flow in the cat were more reproducible than the radioactive microsphere determinations, but the average values obtained with both techniques were similar.

#### B. NMR Studies of Pancreatic Metabolism

Alcoholic pancreatitis is the major source of pancreatitis in the United States, and has a high mortality rate. In the last year, we undertook an in vivo NMR study of energy metabolism in the canine pancreas, with emphasis on the effects of acute pancreatitis. We found that stimulus of the pancreas by agents that cause acute pancreatitis (e.g., caerulein), or infusion of small amounts of oleic acid, which models the hyperlipidemia associated with acute alcoholic pancreatitis, cause a rapid decline in high-energy phosphates, which could be correlated with secretory output, vascular resistance, edema and oxygen consumption.

#### C. Studies of Relationship Between Membrane Surface Charge Density and Surface Potential

One of our long-term goals is to determine the relationship between the structure of charges at the surface of a biological membrane and the electrostatic surface potential. We previously determined that charges extending approximately 10 angstroms from the surface (i.e., sialic acid residue in gangliosides) have a different effect on the surface potential than charges located directly at the surface (i.e., anionic phospholipids). In the last year, we developed a procedure for the asymmetric incorporation of purified glycoporphin - a sialoglycoprotein isolated from the erythrocyte membrane - into unilamellar phospholipid bilayer vesicles. In the coming year we will use these reconstituted vesicles to determine the effect of the sialic acid residues in glycoporphin, which extend up to 100 angstroms from the surface, on the surface potential of bilayer membranes.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 00001-02 LMMB
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Effects of Ethanol on Gastrointestinal Biochemistry and Physiology		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	M.-T. Huang	Chemist LMMB, NIAAA
Other:	R.L. Veech	Acting Chief LMMB, NIAAA
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Metabolism and Metabolic Biology		
SECTION Metabolic Control		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.8	0.2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)  <p>             A surgical method was developed to cannulate chronically both the portal and hepatic veins of laboratory rats. This experimental system is useful for studies on intestinal absorption and hepatic extractions of nutrients. With this experimental system, the following objectives can be determined (1) the effect of ethanol on GI absorption and liver metabolism and (2) glucose paradox. In the first study, the rate of ethanol elimination will be determined in rats meal-fed with diet containing glucose, fructose, mixture of glucose and fructose, and sucrose to determine the importance of alcohol dehydrogenase and redox state in liver on the metabolism of ethanol <u>in vivo</u>. In the second study, portal-hepatic difference of glucose and gluconeogenic precursors will be determined in order to resolve the paradox that liver can not utilize glucose efficiently. Our results, in the latter subject, show that liver can utilize exogenous glucose and can synthesize glycogen directly from exogenous glucose directly. Recent theory on the pathway of glycogen synthesis in liver (Glucose-C3-G6P-Glucogen) was found to be based on questionable data and inadequate method of calculation.           </p>		

Project Description:Investigators:

M.T. Huang  
R.L. Veech

Chemist  
Acting Chief

LMMB, NIAAA  
LMMB, NIAAA

Objectives:

Accessibility to the portal vein of the common laboratory rat has been a serious experimental obstacle. Because of its location in between gastrointestinal tract and liver, its constituents are not expected to be similar to arterial or venous circulation. For studies on intestinal absorption of nutrients, monitoring changes of concentrations in systemic circulation can produce serious artefact, if liver can sequester the nutrient. We, therefore, decided to develop a chronic cannulation method for the portal vein. The experimental system enables the studies on the intestinal absorption of nutrient in the conscious rats and on the hepatic extraction by measuring portal-hepatic difference of the nutrient.

Two major problems can be studied immediately following the development of the technique. One is on whether liver can utilize glucose. This is pertinent to the so-called "glucose paradox" of Katz and McGarry who concluded that liver can not utilize glucose. Glycogen synthesis in liver is coming from gluconeogenesis, the indirect pathway. The second problem is on the metabolism of ethanol *in vivo*. It has been suggested that the rate of ethanol metabolism is related to the turnover of cytosolic ATP and redox states of the liver. Cornell, however, concluded that liver alcohol dehydrogenase determines the rate of ethanol metabolism. The rate of alcohol dehydrogenase calculated from a rate equation for a ordered be-be reaction and the kinetic constants of the enzyme coincide with the measured rate of ethanol metabolism. The effect of cytosolic redox state on the metabolism of ethanol is evident only when the intermediates of the malate-aspartate shuttle is artefactually depleted, such as in isolated hepatocytes. Ethanol concentrations in the portal and hepatic vein will be studied in rats meal-fed diet containing different carbohydrates. The intestinal absorption and hepatic metabolism of ethanol can thus be studied.

Methods Employed:

**Cannulation:** Rats were anesthetized with pentobarbital. Leptotomy was performed with 5 cm longitudinal incision in the abdominal walls. Duodenum was retracted to expose the inferior pancreato-duodenal vein. A PE10 cannula was inserted from pancreato-duodenal vein to the portal vein. The hepatic vein was cannulated through the external jugular vein. The length of the cannula to be inserted was premeasured so that the tip of cannula was placed just below the diaphragm. The cannula were exteriorized in the back of the animal. After the surgery, animals were housed individually.

**Meal-feeding:** Meal-feeding was started on the second day of the surgery from 9:00 am to 11:00 am for a total period of seven days. Diets were prepared by mixing 50% grounded chow with 50% glucose, 50% fructose, 50% sucrose or 25% glucose + 25% fructose. Body weight and daily intake of diet were recorded. Blood were sampled from the portal and hepatic cannula (0.3 ml each) before and after meal fed on the 1st, 3rd, 5th, and 7th day.

**Metabolite assay:** Blood plasma was separated from blood cells by centrifugation. Aliquots (20 ul) of plasma were precipitated by sulfosalicylic acid and derivatized with o-phthalaldehyde in the presence of 3-mercaptopropionic acid. Reverse phase HPLC on C-18

column were used to quantitate the amino acid content of the plasma. The concentration of glucose, fructose, lactate, and pyruvate were measured enzymatically from another aliquot of plasma (0.1 ml) prepared by 3.6% PCA and neutralization.

**The metabolism of ethanol:** On the seventh day after the end of meal feeding, the rats were briefly anesthetized with ether. A dose of ethanol (5 u; 1 ml/100 g 6.w) was intubated. Blood was sampled through the portal and hepatic vein at 1, 5, 20, and 40 min after the intubation. Ethanol was assayed enzymatically with alcohol dehydrogenase.

#### Major Findings:

**Rats:** Effects of surgery on body wt and food intake were followed for a period of seven days. Body weight decrease substantially ( 8%) on the first day post-surgically. Thereafter, the rate of decrease of body weight alleviated. On the third or fourth day after the surgery, body weight of the animal stabilized. A slight increase in body weight was observed on the sixth or seventh days after the surgery. No food intake was observed on the first and second day after the surgery. On the third day, the amount of diet consumed was 5 g, regardless of the composition of the diet. From the fourth day on, daily food intake increased to above 10 g per day.

**Hepatic-portal difference of glucose concentration:** Glucose concentration in plasma of portal and hepatic blood was determined. It was found that in fasted state liver generally released glucose as the glucose concentration in the hepatic vein was found to be higher than that in the portal vein. After meal, hepatic-portal difference of glucose concentration seems to correlate with the glucose concentration in the portal vein, with crossover point at a portal concentration of 8 mM. Above this level, net extraction of glucose can be seen in fructose- and glucose-supplemental rats. Thus, it seems that liver can utilize glucose, but only when the concentration in the portal vein is raised above 8 mM. The significance of this finding to "glucose paradox" has to be studied further. The hepatic extraction can be converted to the rate of glucose utilization if the rate of blood flow in liver is known.

**Metabolism of ethanol *in vivo*:** In one study with a glucose-fed and fructose-fed rat, the rate of disappearance of orally ingested ethanol in hepatic blood are not significantly different. However, the concentration of ethanol in the two rats were not the same. Further studies are necessary to determine the effect of dietary carbohydrate on the absorption of ethanol and liver extraction of ethanol.

#### Significance to Biomedical Research and the Program of the Institute:

Present work describe a method to open up the portal vein of laboratory rat for blood sampling and injection. Studies involving this experimental system have been initiated to resolve the issue of glucose paradox, whether liver can utilize glucose or the effect of fructose on the metabolism of ethanol.

#### Proposed Course:

In the coming year, the effect of ethanol on the biochemistry and physiology of GI will be studied.

Publications:

None.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

Z01 AA 00032-04 LMMB

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification of Glucose Metabolites in Brain and the Relationship to 2-Deoxyglucose Method

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: M.-T. Huang

Chemist

LMMB, NIAAA

Others: R.L. Vecch

Acting Chief

LMMB, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Metabolic Control

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

1.75

1.0

0.75

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The magnitude of the dephosphorylation of glucose-6-phosphate in brain *in vivo* was re-examined in this report. Qualitatively, the reaction was examined by comparing the ratio of 3H-glucose/14C-glucose in brain and blood from 1 to 10 min after an injection of [2-3H]- and [U-14C]-glucose. Current findings show that our previous result of a 35% decrease in 3H/14C ratio in brain glucose 5 minutes after the injection was caused by undetected contaminants coeluted with glucose in the isolation process. Using an improved fractionation procedure, in the present study, we found that the rate of decrease of the 3H/14C ratio in brain glucose is significantly greater than that in blood glucose; the difference in slopes of the two time-courses was 29%. Quantitatively, the dephosphorylation of glucose-6-phosphate was studied from data obtained within the first minute after the injection of tracers. The formation of radioactive metabolites from [2-3H]-glucose is significantly greater than from [U-14C]-glucose immediately following the injection, 25% at 10 s, 21% at 20 s, and 14% at 30 s after the injection. The decreasing trend is caused by loss of tritiated water to blood circulation. The rate of glucose utilization calculated from the initial rates of formation of radioactive metabolites from [2-3H]-glucose was 1.04  $\mu\text{mol}/\text{min}/\text{g}$ ; from [U-14C]-glucose was 0.83  $\mu\text{mol}/\text{min}/\text{g}$ . The difference of 0.21  $\mu\text{mol}/\text{min}/\text{g}$  was attributed to the dephosphorylation of glucose-6-phosphate in brain. Current findings reaffirm our previous conclusion that in brain, the dephosphorylation of glucose-6-phosphate is active. Because of rapid rate of glucose turnover in brain and the rapid equilibration of labeled glucose in the body aided by blood circulation, data obtained 2 min after the injection as reported by Nelson *et al.* (Nelson, T., Lucignani, G., Atlas, S., Crane, A.M., Dienel, G.A., and Sokoloff, L.: Re-examination of glucose-6-phosphatase activity in brain *in vivo*: No evidence for a futile cycle. *Science* 229: 60-62, 1985) are not sufficient to conclude the quantitative aspect of the dephosphorylation of glucose-6-phosphate.

This project has been terminated.

Project Description:Investigators:

M.-T. Huang	Chemist	LMMB, NIAAA
R.L. Veech	Acting Chief	LMMB, NIAAA

Objectives:

We have previously studied substrate cycling between glucose and glucose-6-phosphate in brain *in vivo* by comparing the metabolic rates of [2-<sup>3</sup>H]-glucose and [U-<sup>14</sup>C]-glucose (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. *J Biol Chem* 257: 11358-11363, 1982). The tracers were injected as a bolus through the internal carotid artery to brain (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. *J Biol Chem* 257: 11358-11363, 1982). The metabolic properties of these two tracers are expected to be identical on passage through the blood brain barrier and during phosphorylation by hexokinase. Only after the formation of glucose-6-phosphate, the metabolic fates of the two tracers differ. During the formation of fructose-6-phosphate from glucose-6-phosphate, tritium radioactivity is immediately released from the carbon skeleton. Since glucose-6-phosphate and fructose-6-phosphate reach equilibrium readily, glucose-6-phosphate in brain is expected to retain only the <sup>14</sup>C radioactivity. A time-dependent decrease in the <sup>3</sup>H/<sup>14</sup>C ratio in tissue glucose is indicative for the resynthesis of glucose from the dephosphorylation of glucose-6-phosphate (Katz, J., Wals, P.A. and Rognstad, R.: Glucose phosphorylation, glucose-6-phosphatase, and recycling in rat hepatocytes. *J Biol Chem* 253: 4530-4536, 1978). In our previous study (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. *J Biol Chem* 257: 11358-11363, 1982), the decrease in the <sup>3</sup>H/<sup>14</sup>C ratio in brain glucose was followed for a period of 10 min and the data was cited as a qualitative evidence for the dephosphorylation of glucose-6-phosphate in brain. Quantitatively, the extent of the dephosphorylation of glucose-6-phosphate was concluded from data depicting the early minute changes of the injected tracers (Huang, M.-T. and Veech, R.L. (1982) The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. *J Biol Chem* 257: 11358-11363, 1982).

The results and conclusion of our previous study (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. *J Biol Chem* 257: 11358-11363, 1982) were challenged recently by Nelson *et al.* (Nelson, T., Lucignani, G., Atlas, S., Crane, A.M., Dienel, G.A., and Sokoloff, L.: Re-examination of glucose-6-phosphatase activity in brain *in vivo*: No evidence for a futile cycle. *Science* 229: 60-62, 1985). These investigators repeated our experiment following our experimental protocol and found that glucose isolated from the anion exchange resin in borate form, as we have reported (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. *J Biol Chem* 257: 11358-11363, 1982), was contaminated. They measured the <sup>3</sup>H/<sup>14</sup>C ratio of the purified glucose in brain from 2 to 10 min and found that the time-course was not significantly different from that of blood glucose. The quantitative aspect of the dephosphorylation of glucose-6-phosphate which was concluded from data obtained from the early minute samples in our previous report was not discussed in the report of Nelson *et al.* (Nelson, T., Lucignani, G., Atlas, S., Crane, A.M., Dienel, G.A., and Sokoloff, L.: Re-examination of glucose-6-phosphatase activity in brain *in vivo*: No evidence for a futile cycle. *Science* 229: 60-62, 1985). Nevertheless, these authors concluded that the magnitude of the dephosphorylation of glucose-6-phosphate in brain *in vivo* reported by us was unfounded (Nelson, T., Lucignani, G., Atlas, S., Crane, A.M., Dienel, G.A., and Sokoloff, L.: Re-examination of glucose-6-phosphatase activity in brain *in vivo*: No evidence for a futile cycle. *Science* 229: 60-62, 1985).



In this report, the possible presence of impurities in radioactive glucose isolated from the borate form of the anion exchange resin was examined. The metabolic properties of  $[2\text{-}^3\text{H}]$ - and  $[\text{U-}^{14}\text{C}]$ -glucose in brain during the first minute after the injection of the tracers were also determined to calculate initial rates of glucose phosphorylation. The difference in the initial rate of glucose phosphorylation measured from these two tracers in turn yields information on the quantitative aspect of the dephosphorylation of glucose-6-phosphate.

#### Methods Employed:

Rats were cannulated in internal carotid arteries as previously described (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. *J Biol Chem* 257: 11358-11363, 1982). Rat brains were obtained by freeze-blowing method (Veech, R.L., Harris, R.L., Veloso, D. and Veech, E.H.: Freeze-blowing: A new technique for the study on brain *in vivo*. *J Neurochem* 20: 183-188, 1973) at various times after the injection of  $[2\text{-}^3\text{H}]$ ,  $[\text{U-}^{14}\text{C}]$ -glucose (100 uCi/10 uCi; New England Nuclear) into cerebral circulation through the implanted cannula.

Perchloric acid (3.6%) extract of frozen brains were neutralized with 30% KOH. Radioactive metabolites of glucose in the brain extract were fractionated according to their charges and functional groups. Neutral, cationic and anionic metabolites of glucose in brain extract were fractionated by column chromatography on Dowex AG 1 x 8 formate resin (BioRad) and Dowex 50 W x 8<sup>+</sup> resins (BioRad) arranged in sequence. Neutral metabolites were eluted from the two columns in 10 ml of water. Cationic metabolites were eluted from Dowex 50 (H<sup>+</sup>) column with 10 ml 1 N H<sub>4</sub>OH and anionic metabolites were eluted from the Dowex AG 1 x 8 formate column with 5 ml 1 N HCl. Radioactive metabolites in neutral, anionic and cationic fraction were further separated on high performance liquid chromatography (HPLC). Brain samples for HPLC analysis were obtained from rats injected with 100 uCi  $[\text{U-}^{14}\text{C}]$ -glucose through the internal carotid artery.

The HPLC system consists of a M-45 pump (Waters Associates), WISP 710B injector (Waters Associates), and HPX 87C column (BioRad). The column was operated at 85°C. For the analysis of radioactive metabolites in the eluates of the cation exchange column, HPX 87C columns was eluted with 10 mM Tris buffer (pH 9.0) at a flow rate of 1 ml/min. Eluates from the column were collected in 1 ml fractions. Radioactivity in each fraction was determined by liquid scintillation counter (Beckman) to construct the chromatogram. Metabolites in the eluates resolved by HPLC were analyzed by mass spectrometry (Voyksner, R.D., Bursey, J.T., and Pellizzari, E.D.: Postcolumn addition of buffer for thermospray liquid chromatography/mass spectrometry identification of pesticides. *Anal Chem* 56: 1507-1514, 1984) to determine their identities. The LC/MS system consists of the HPLC system described above, a Finnigan 4500 quadrupole mass spectrometer and a thermospray interface (Finnigan MAT Co., San Jose, CA). The thermospray interface consisted of a vaporizer, an aerosol and an ionization assemble. Materials in the effluent of the HPLC column are ionized and detected in the mass spectrometer which was scanned from m/e 70 to 400 at 2 seconds per scan. The data were collected and processed with an INCOS data system (Finnigan MAT Co., San Jose, CA). Alternatively, glucose was separated by anion exchange resin (Dowex AG x 1) in borate and formate forms as previously described (Huang and Veech, 1982). Brain extracts were subjected to column chromatography with anion exchange resin in formate form to remove anionic metabolites (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. *J Biol Chem* 257: 11358-11363, 1982). Eluates from the borate column were fractionated on cation exchange resin. Radioactivity retained by the cation exchange resin was analyzed by LC/MS method as described.

Paper chromatography was carried out for various glucose-containing fractions. Carrier glucose (1  $\mu$ mol) was co-spotted with the sample on a 50 cm strip of Whatman No. 1 paper. The descending paper chromatogram was developed in a solvent system consisting of n-butanol: acetic acid: water (4:1:5). The Rf of glucose was determined by autoradiographic method or by color reaction using sodium periodate and benzidine chloride (Cifonelli, J.A. and Smith, F.: Anal Chem 26: 1132, 1954). Radioactivities in spots corresponding to glucose were eluted four times with 2 ml 0.1 N HCl from the paper. Solvent in various glucose-containing fractions was evaporated and the radioactivity determined by external standard method.

### Major Findings:

**Non-glucose radioactive metabolites in the eluates of the borate column:** Compounds with vicinyl diols form negatively charged complexes with borate which can be retained by anion exchange resin. Fractionation of radioactive metabolites in brain extract by tandem columns of anion exchange resin, Dowex AG 1 x 8 in formate (formate column) and borate forms (borate column), yields radioactive glucose in the eluates of the borate resin column (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. J Biol Chem 257: 11358-11363, 1982). When the eluates of the borate column was chromatographed on cation exchange resin, significant radioactivities was retained by the resin. This result thus confirms the observation of Nelson *et al.* (Nelson, T., Lucignani, G., Atlas, S., Crane, A.M., Dienel, G.A., and Sokoloff, L.: Re-examination of glucose-6-phosphatase activity in brain *in vivo*: No evidence for a futile cycle. Science 229: 60-62, 1985), that the radioactivities in eluates of the borate column contains non-glucose radioactive entities. The chromatographic properties of the impurities with cation exchange resin indicated that these compounds are positively charged and can be eluted from the cation exchange resin by 10 ml 1 N  $\text{NH}_4\text{OH}$ . The time-course of the formation of these cationic metabolites was shown in Figure 1. Initially, the content of these cationic metabolites in brain extract is low. The level gradually increases and approaches that of radioactive glucose at about 10 min after the injection.

These impurities can be resolved into two major peaks by HPLC on HPX 87C column eluted with 10 mM Tris buffer at pH 9.0. The retention time of the first peak was 14 min and the second was 21 min which were identical to standard glutamine and  $\gamma$ -aminobutyric acid (GABA), respectively. The identities of the two radioactive metabolites as glutamine and GABA were further confirmed by their mass spectra obtained from liquid chromatography-mass spectrometry (LC/MS).

**$^3\text{H}/^{14}\text{C}$  ratio in brain glucose:** A more stringent method using two column chromatographic steps for the fractionation of radioactive metabolites and purification of radioactive glucose in brain extract was adopted. Tandem columns of anion exchange resin in formate and borate forms were used initially to obtain tritiated water, anionic metabolites and compounds retainable by the borate column. The latter were subsequently eluted from the column by 4 N formic acid. Formic acid and borate residues were removed by evaporating in methanol at 50°C under a stream of  $\text{N}_2$ . The remainders were subjected to cation exchange chromatography to separate cationic impurities from glucose.

The slope of the time-course for the decrease in  $^3\text{H}/^{14}\text{C}$  ratio in brain glucose is not as great as we have previously reported (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. J Biol Chem 257: 11358-11363, 1982). Nevertheless, the slope of the time dependent decrease in  $^3\text{H}/^{14}\text{C}$  ratio in brain glucose is still greater (29%) than that found for blood glucose. The ratio of  $^3\text{H}/^{14}\text{C}$  in brain glucose and blood glucose at 10 min after the injection were significantly different ( $p < 0.05$ ). Further purification with paper chromatography did not affect the time-course of the decrease in  $^3\text{H}/^{14}\text{C}$  in brain glucose.

Metabolic changes for [2-<sup>3</sup>H]-glucose and [U-<sup>14</sup>C]-glucose during the first minute: The metabolic changes for [2-<sup>3</sup>H]- and [U-<sup>14</sup>C]-glucose within the first minutes after the injection, which were not mentioned by Nelson *et al.* (Nelson, T., Lucignani, G., Atlas, S., Crane, A.M., Diemel, G.A., and Sokoloff, L.: Re-examination of glucose-6-phosphatase activity in brain *in vivo*: No evidence for a futile cycle. *Science* 229: 60-62, 1985) in their report, were also examined. Radioactive metabolites were fractionated by the two-step, three-column chromatographic method. Radioactive products of glycolysis for [2-<sup>3</sup>H]-glucose were found in the tritiated water, anionic metabolites and cationic metabolite fractions; radioactive products of glycolysis for [U-<sup>14</sup>C]-glucose were found in the anionic and cationic products fraction. Contents of radioactive products of glycolysis from [2-<sup>3</sup>H]- and [U-<sup>14</sup>C]-glucose were compared. The <sup>3</sup>H/<sup>14</sup>C ratios in metabolic products arising from glucose phosphorylation were significantly elevated compared to the ratio in the precursor glucose during the first 30 seconds after the injection, indicating excess formation of products from [2-<sup>3</sup>H]-glucose than from [U-<sup>14</sup>C]-glucose. The excess production of <sup>3</sup>H- over <sup>14</sup>C-metabolites was 25% at 10 sec, 21% at 20 sec and 14% at 30 sec after the injection. The decreasing trend was caused by the release of tritiated water and, therefore, loss of tritium labeled glycolytic products from brain tissue (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. *J Biol Chem* 257: 11358-11363, 1982).

Incubation with yeast hexokinase and phosphoglucose isomerase did not cause the <sup>3</sup>H/<sup>14</sup>C ratio in products of glucose phosphorylation to be different from the ratio in glucose, indicating that the excess rate of metabolism of [2-<sup>3</sup>H]-glucose observed *in vivo* is not caused by the isotope effect in the phosphorylation and detritiation processes. The excess formation of tritium labeled products of hexokinase in brain indicates that the initial rate of glucose metabolism measured from [2-<sup>3</sup>H]-glucose is significantly greater than from [U-<sup>14</sup>C]-glucose.

The initial rates of metabolism of [2-<sup>3</sup>H]- and [U-<sup>14</sup>C]-glucose: The time-courses for the formation of tritiated water and labeled anionic metabolites in brain during the first minute after the injection were determined. The appearances of <sup>3</sup>H and <sup>14</sup>C radioactivity in the cationic fraction were not shown because of low level of radioactivity in the initial period immediately following the injection of labeled glucose. The formation of tritiated water and labeled anionic metabolites increase linearly during the first 20 seconds and leveled off thereafter, reflecting the reduction of radioactivity in the precursor pool. The initial rates of formation of labeled metabolites (dpm/g/min) were calculated from the slope of the linear segment of the time-course. The rates of phosphorylation of labeled glucose were calculated by dividing the initial rates of formation of labeled products (dpm/g/min) by the specific activity of labeled glucose (dpm/mol) derived by extrapolation to zero time. The initial rate of glucose phosphorylation thus calculated was 1.04  $\mu$ mol/g/min from [2-<sup>3</sup>H]-glucose and 0.83  $\mu$ mol/g/min from [U-<sup>14</sup>C]-glucose. The difference in the rate of glucose phosphorylation from these two tracers was 0.21  $\mu$ mol/g/min.

#### Significance to Biomedical Research and the Program of the Institute:

In a recent study, Nelson *et al.* (Nelson, T., Lucignani, G., Atlas, S., Crane, A.M., Diemel, G.A., and Sokoloff, L.: Re-examination of glucose-6-phosphatase activity in brain *in vivo*: No evidence for a futile cycle. *Science* 229: 60-62, 1985) examined the <sup>3</sup>H-glucose/<sup>14</sup>C-glucose ratio in rat brain from 2-8 min after an intraarterial injection of [2-<sup>3</sup>H], [U-<sup>14</sup>C]-glucose by an experimental protocol essentially similar to what we have previously reported (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain, *J Biol Chem* 257: 11358-11363, 1982). Although they observed a time-dependent decrease in <sup>3</sup>H/<sup>14</sup>C ratio in the eluates of the borate column, they attributed this to contaminants rather than to glucose itself. When the eluates of the Dowex AG 1 x 8 borate column were chromatographed on a cation exchange column, up to 45% of radioactivity

was removed. Our current result support the finding of Nelson *et al.* (Nelson, T., Lucignani, G., Atlas, S., Crane, A.M., Diemel, G.A., and Sokoloff, L.: Re-examination of glucose-6-phosphatase activity in brain *in vivo*: No evidence for a futile cycle. *Science* 229: 60-62, 1985) that our previously reported decrease in the  $^3\text{H}/^{14}\text{C}$  ratio in brain glucose was exaggerated by contaminants which carry mainly  $^{14}\text{C}$ -radioactivity. However, after these impurities were removed by cation exchange resin, we still found that the decrease in  $^3\text{H}/^{14}\text{C}$  ratio in brain glucose is greater than blood glucose. The impurities were identified in the current study as glutamine and  $\alpha$ -aminobutyric acid, rather than fructose or glucosamine as suggested by Nelson *et al.* (Nelson, T., Lucignani, G., Atlas, S., Crane, A.M., Diemel, G.A., and Sokoloff, L.: Re-examination of glucose-6-phosphatase activity in brain *in vivo*: No evidence for a futile cycle. *Science* 229: 60-62, 1985).

Nelson *et al.* concluded, from the time-course of the  $^3\text{H}/^{14}\text{C}$  in brain glucose and blood glucose, that the rate of the dephosphorylation of glucose-6-phosphate in brain *in vivo* is insignificant (Nelson, T., Lucignani, G., Atlas, S., Crane, A.M., Diemel, G.A., and Sokoloff, L.: Re-examination of glucose-6-phosphatase activity in brain *in vivo*: No evidence for a futile cycle. *Science* 229: 60-62, 1985; Nelson, T., Lucignani, G., Gooch, J., Crane, A.M., and Sokoloff, L.: Invalidity of criticisms of the 2-deoxyglucose method based on alleged glucose-6-phosphatase activity in brain. *J Neurochem* 46: 905-919, 1986; Nelson, T., Lucignani, G., and Sokoloff, L.: Measurement of brain deoxyglucose metabolism by NMR. *Science* 232: 776, 1986). The data they presented, however, were not sufficient to support their conclusion. The experimental protocol for the injection through the internal carotid artery was designed to confine the injected tracers and their metabolic transformation in brain, mimicking *in vitro* perfusion of isolated organ. The rapid rate of glucose turnover in brain and blood circulation in rats dictate the short time span for the objective of this experimental protocol to be fulfilled. In our previous study, we have found that the specific activity of glucose in the systemic circulation and cerebral circulation equilibrated in two minutes after the injection, and that the appearance of tritiated water in cerebral circulation from the catabolism of  $[2\text{-}^3\text{H}]\text{-glucose}$  also reached a maximum in two minutes. For these reasons, quantitation of the rate of glucose phosphorylation and the rate of glucose-6-phosphate dephosphorylation *in vivo* are not possible from data collected two minutes after the injection. Our earlier conclusion on the extent of the dephosphorylation of glucose-6-phosphate was drawn from the difference in the initial rates of metabolism of  $[2\text{-}^3\text{H}]\text{-}$  and  $[\text{U-}^{14}\text{C}]\text{-glucose}$  (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. *J Biol Chem* 257: 11358-11363, 1982). Without data quantitating the metabolic change of  $[2\text{-}^3\text{H}]\text{-}$  and  $[\text{U-}^{14}\text{C}]\text{-glucose}$  immediately following the injection, any conclusion on the rate of dephosphorylation of glucose-6-phosphate is unwarranted.

More recently, several reports from Sokoloff's group defending the validity of the 2-deoxyglucose method were published (Nelson, T., Lucignani, G., Gooch, J., Crane, A.M., and Sokoloff, L.: Invalidity of criticisms of the 2-deoxyglucose method based on alleged glucose-6-phosphatase activity in brain. *J Neurochem* 46: 905-919, 1986; Nelson, T., Lucignani, G., and Sokoloff, L.: Measurement of brain deoxyglucose metabolism by NMR. *Science* 232: 776, 1986). In one report (Nelson, T., Lucignani, G., Gooch, J., Crane, A.M., and Sokoloff, L.: Invalidity of criticisms of the 2-deoxyglucose method based on alleged glucose-6-phosphatase activity in brain. *J Neurochem* 46: 905-919, 1986). They attributed our previous result of a rate of 4.5%/min for the dephosphorylation of 2-deoxyglucose phosphate in brain *in vivo* to widely scattered data and the selection of a model with a predetermined parameter for the dephosphorylation of 2-deoxyglucose phosphate. The scattering of data may be inevitable in the *in vivo* experiments as was also shown by Nelson *et al.*, that the mean for brain 2-deoxyglucose phosphate can vary as much as 50% under the influence of factors such as blood sampling which is unrelated to brain energy metabolism (see Fig. 2a and Fig. 2, of report of Nelson, T., Lucignani, G., Gooch, J., Crane, A.M., and Sokoloff, L.: Invalidity of criticisms of the 2-deoxyglucose method based on alleged glucose-6-phosphatase activity in

brain. *J Neurochem* 46: 905-919, 1986). We have also estimated the rate constant for the dephosphorylation of 2-deoxyglucose-6-phosphate by following the decay of [ $^{14}\text{C}$ ]-2-deoxyglucose phosphate in brain, without invoking any kinetic modeling (see Fig. 2 of Huang, M.-T. and Veech, R.L.: Metabolic fluxes between [ $^{14}\text{C}$ ]-deoxyglucose and [ $^{14}\text{C}$ ]-2-deoxyglucose-6-phosphate in brain *in vivo*. *J Neurochem* 44: 556-573, 1985).

The enzymic apparatus for the dephosphorylation of glucose-6-phosphate or 2-deoxyglucose-6-phosphatase were not specified in our previous works (Huang and Veech, 1982; Huang, M.-T. and Veech, R.L.: Metabolic fluxes between [ $^{14}\text{C}$ ]-deoxyglucose and [ $^{14}\text{C}$ ]-2-deoxyglucose-6-phosphate in brain *in vivo*. *J Neurochem* 44: 556-573, 1985). Nelson *et al.*, while maintaining that the dephosphorylation of hexose-6-phosphates is insignificant, seem to acknowledge the activity of glucose-6-phosphatase (Nelson, T., Lucignani, G., Gooch, J., Crane, A.M., and Sokoloff, L.: Invalidity of criticisms of the 2-deoxyglucose method based on alleged glucose-6-phosphatase activity in brain. *J Neurochem* 46: 905-919, 1986; Nelson, T., Lucignani, G., and Sokoloff, L.: Measurement of brain deoxyglucose metabolism by NMR. *Science* 232: 776, 1986). They frequently alluded to a recent report showing that a translocase for glucose-6-phosphate is lacking. They concluded that it is necessary to take 45 min for hexose-6-phosphates to migrate to the luminal side of the endoplasmic reticulum to be hydrolyzed by glucose-6-phosphatase, (Nelson, T., Lucignani, G., Gooch, J., Crane, A.M., and Sokoloff, L.: Invalidity of criticisms of the 2-deoxyglucose method based on alleged glucose-6-phosphatase activity in brain. *J Neurochem* 46: 905-919, 1986; Nelson, T., Lucignani, G., and Sokoloff, L.: Measurement of brain deoxyglucose metabolism by NMR. *Science* 232: 776, 1986) without any supporting evidence. Deuel *et al.* (1985) recently determined a half-life of decay of 120 min for 2-deoxyglucose phosphate in brain by nuclear magnetic resonance after injecting intravenously a pharmacological dose of [ $^{31}\text{P}$ ]-2-deoxyglucose. This evidence was cited by Nelson *et al.* (Nelson, T., Lucignani, G., and Sokoloff, L.: Measurement of brain deoxyglucose metabolism by NMR. *Science* 232: 776, 1986) to support the 45 min lag time for the dephosphorylation of radioactive 2-deoxyglucose-6-phosphate in brain. They noted the similarity in the time-course of [ $^{31}\text{P}$ ]-2-deoxyglucose-6-phosphate reported by Deuel *et al.* (Deuel, R.K., Yue, G.M., Sherman, W.R., Schnicker, D.J., Ackerman, J.J.H.: Monitoring the time course of cerebral deoxyglucose metabolism by  $^{31}\text{P}$  nuclear resonance spectroscopy. *Science* 228: 1229-1132, 1985) and that of cerebral glucose utilization rates reported by Sokoloff *et al.* (1982) using tracer amount of [ $^{14}\text{C}$ ]-2-deoxyglucose. This similarity in the rates for tracer dose and pharmacological dose is contradictory to the kinetic properties of glucose-6-phosphatase of reported a  $K_m$  of 8-9 mM for brain glucose-6-phosphatase. Both the data of Deuel *et al.* (Deuel, R.K., Yue, G.M., Sherman, W.R., Schnicker, D.J., Ackerman, J.J.H.: Monitoring the time course of cerebral deoxyglucose metabolism by  $^{31}\text{P}$  nuclear resonance spectroscopy. *Science* 228: 1229-1132, 1985; Sokoloff, L., Rejch, M., Kennedy, C., Des Rosiers, N.H., Patlak, C.S., Pettigrew, K.D., Sakurada, O., and Shirohara, M.: The [ $^{14}\text{C}$ ]-deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure and the normal value in conscious and anesthetized albino rat. *J Neurochem* 28: 897-916, 1977; Nelson, T., Lucignani, G., and Sokoloff, L.: Measurement of brain deoxyglucose metabolism by NMR. *Science* 232: 776, 1986) can be interpreted by the traditional two-compartment *in vivo* kinetics with continuous formation and disappearance of products (Shipley, R.A., and Clark, R.E. (1972) Tracer methods for *in vivo* kinetics. Theory and applications. Academic Press, New York and London).

The disagreement and controversy surrounding the 2-deoxyglucose method highlighted the complicated nature of the theoretical basis for the 2-deoxyglucose method (Fox, J.L.: PET scan controversy aired. *Science* 224: 143-144, 1984; Cunningham, V.J. and Cremer, J.E.: Current assumptions behind the use of PET scanning for measuring glucose utilization in brain. *Trend Neuro Sci* 8: 96-99, 1985). It has been known that on transport through the blood brain barrier, a larger fraction of labeled 2-deoxyglucose than radioactive glucose is taken up by the brain (Huang, M.-T. and Veech, R.L.: Metabolic fluxes between [ $^{14}\text{C}$ ]-deoxyglucose and [ $^{14}\text{C}$ ]-2-deoxyglucose-6-phosphate in brain *in vivo*. *J Neurochem* 44: 556-573, 1985). This may indicate

that glucose carriers on the blood brain barrier may not be of a single entity and may consist of several isozymes with high and low  $K_m$ 's. Since the lumped constant of the 2-deoxyglucose method was determined from the difference in transport and phosphorylation between glucose and 2-deoxyglucose under a certain physiological state, its universal applicability has been questioned (Crane, P.D., Pardridge, W.M., Brann, L.D., Nyerges, A.M., and Oldendorf, W.M.: The interaction of transport and metabolism on brain glucose utilization: A re-evaluation of the lumped constant. J Neurochem 36: 1601-1604, 1981; Pardridge, W.M., Crane, P.D., and Oldendorf, W.H.: On "Lumped constant" normograms. J Neurochem 39: 1775-1776, 1982) and has been shown to produce misleading results (Takei, H., Fredericks, W.R., and Rapoport, S.I.: The lumped constant in the deoxyglucose procedure declines with age in Fischer 344 rats. J Neurochem 46: 931-938, 1986).

Our current study showed a 25% difference in the rates of glucose phosphorylation measured with  $[2-^3\text{H}]\text{-glucose}$  and  $[\text{U-}^{14}\text{C}]\text{-glucose}$ . The rate estimated from  $[2-^3\text{H}]\text{-glucose}$ , representing metabolic flux through hexokinase is 1.04  $\mu\text{mol/min/g}$ ; while the rate estimated from  $[\text{U-}^{14}\text{C}]\text{-glucose}$ , representing the overall glycolytic flux, is 0.83  $\mu\text{mol/min/g}$ . The difference, 0.21  $\mu\text{mol/min/g}$ , can only be interpreted as the rate of dephosphorylation of glucose-6-phosphate. In our previous study (Huang, M.-T. and Veech, R.L.: The quantitative determination of the *in vivo* dephosphorylation of glucose-6-phosphate in rat brain. J Biol Chem 257: 11358-11363, 1982), the apparent rate of glucose phosphorylation was calculated by dividing the content of radioactive products of glucose phosphorylation by the specific activity of glucose and the time span between the injection and brain blowing. The difference in the apparent rate of glucose phosphorylation estimated from  $[2-^3\text{H}]\text{-}$  and  $[\text{U-}^{14}\text{C}]\text{-glucose}$  was 35% in our previous study. In conclusion, both our current and earlier studies show that in brain, the rate for the dephosphorylation of glucose-6-phosphate is significant. The physiological significance of the dephosphorylation of glucose-6-phosphate may be to fine tune the flux through hexokinase in accordance with the overall glycolytic flux.

#### Proposed course:

The universality of the lumped constant will be determined by comparing the metabolic properties of  $[\text{l-}^{14}\text{C}]\text{-2-deoxyglucose}$  and  $[6-^3\text{H}]\text{-glucose}$  in various physiological states. The project is terminated.

#### Publications:

1. Huang, M.T. and Veech, R.L.: Glucose-6-phosphatase activity in brain. Science 234: 1128-1129, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER <b>Z01 AA 00035-01 LMMB</b>
PERIOD COVERED <b>October 1, 1986 to September 30, 1987</b>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Effects of Ethanol and its Metabolites on Metabolism and Inorganic Ion Balance</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	W.L. Gitomer	Senior Staff Fellow LMMB, NIAAA
Others:	R.L. Veech	Acting Chief LMMB, NIAAA
COOPERATING UNITS (if any)  <b>R.L. Ornberg, LCBG, NIDDK; R.D. Leapman, BEI, DRS</b>		
LAB/BRANCH <b>Laboratory of Metabolism and Molecular Biology</b>		
SECTION <b>Metabolic Control</b>		
INSTITUTE AND LOCATION <b>NIAAA, 12501 Washington Ave., Rockville, Maryland 20852</b>		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>It was observed that the treatment of 48hr. starved rats with acetate, propionate or butyrate results in large increases in the hepatic Ca<sup>2+</sup>, Mg<sup>2+</sup> and inorganic pyrophosphate (PPi) content apparently due to the formation of calcium and magnesium PPi precipitates within the mitochondrial matrix. The increase in mitochondrial calcium and magnesium was shown to occur using electron probe x-ray microanalysis. Assuming that the free matrix [Mg<sup>2+</sup>]=1 mM and using the magnesium PPi and calcium PPi solubility products, the free mitochondrial [Ca<sup>2+</sup>] in the liver was calculated to be 1.2 mM after treatment with short chain fatty acids. This observation was then expanded to all metabolic states and it was concluded that under all <u>in vivo</u> conditions thus far studied that, in the <u>in vivo</u> rat liver, calcium and magnesium PPi precipitates are present in the mitochondrial matrix and the free mitochondrial matrix [Ca<sup>2+</sup>] is about 1mM. This value is three orders of magnitude greater than values estimated for the free mitochondrial matrix [Ca<sup>2+</sup>] using isolated mitochondria.</p>		

Project Description:Investigators:

W.L. Gitomer  
R.L. Veech  
R.L. Ornberg  
R.D. Leapman

Senior Staff Fellow  
Acting Chief

LMMB,NIAAA  
LMMB,NIAAA  
LCBG,NIDDK  
BEI,DRS

Objectives:

Ethanol is metabolized in the liver by alcohol dehydrogenase to acetaldehyde and acetaldehyde is further metabolized by aldehyde dehydrogenase to acetate. Most emphasis in the study of the metabolites of ethanol has focused on acetaldehyde. However, the blood concentration of this metabolite rarely exceeds 10uM, while the blood concentration of acetate approaches 2mM even after the consumption of moderate amounts of ethanol. This study was undertaken to elucidate the consequences of acetate metabolism on hepatic inorganic ion content.

Methods Employed:

1. Atomic absorption spectrophotometry for the measurement of inorganic cations.
2. Electron probe x-ray microanalysis for the subcellular localization of inorganic elements.
3. The incubation of isolated hepatocytes under the conditions necessary for the cells to accumulate PPi like the in vivo liver.
4. Enzymatic determination of PPi
5. Dignition fractionation of isolated hepatocytes.

Major Findings:

It has been shown that the treatment of 48hr. starved rats with acetate, propionate or butyrate results in large increases in the hepatic  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and inorganic pyrophosphate [PPi] content apparently due to the formation of calcium and magnesium PPi precipitates within the mitochondrial matrix. From these observations it has been deduced that (1) the free mitochondrial  $[\text{Ca}^{2+}]$  in the liver is 1.2 mM under all in vivo conditions thus far studied, (2) in the in vivo rat liver calcium and magnesium PPi precipitates are present in the mitochondrial matrix, (3) the free energy of the calcium concentration gradient between the mitochondria and the cytosol is equal to the free energy of the cytosolic phosphorylation potential, (4) the gradient of free calcium between the mitochondria and the cytosol is either in near-equilibrium with the mitochondrial membrane potential of -123 mV or the free calcium concentration gradient is the in vivo energy transducer for oxidative phosphorylation, (5) calcium phosphate and carbonate precipitates are likely to occur in the mitochondrial matrix and the physical properties of these inorganic salts set maximum possible values for both the pH within the mitochondria and the pH gradient between the mitochondrial and the cytosol, (6) that the apparent disagreement between the chemical measurements of phosphate in tissue and the measurement of phosphate by NMR is due to the sequestration of phosphate within the mitochondria, and (7) that the concentration of free cytosolic ADP does not regulate the rate of oxygen consumption under most in vivo conditions as proposed by Chance and Williams but that it is regulated by the free cytosolic calcium concentration.



Significance to Biomedical Research and the Program of the Institute

Elevation of intracellular PPi causes some of the largest changes in cellular calcium content yet observed. Intracellular  $\text{Ca}^{2+}$  is one of the major cellular second messengers and in this role, it acts as a regulator of a multitude of metabolic reactions. The role of PPi in regulating cellular  $\text{Ca}^{2+}$  will now have to be considered.

It has been proposed that the activity of the Tricarboxylic Acid Cycle is regulated by micromolar concentrations of  $\text{Ca}^{2+}$  in the matrix of the mitochondria. The finding that the mitochondrial  $\text{Ca}^{2+}$  concentration is 1mM means that the activity of the enzymes in vivo is not regulated by  $\text{Ca}^{2+}$ .

Finally a number of disease states found in alcoholics are related to changes in calcium content of tissues including bone disease. This disease state should now be considered from the point of view of the effects of acetate metabolism on tissue inorganic ion content.

Proposed Course:

Acetate is the major metabolic product of ethanol metabolism in the liver with about 90% of the acetate which is produced during ethanol metabolism and is released from the liver into the general blood supply. This large "export" of acetate from the liver would be expected to have a profound effect on the inorganic ion balance of a number of different tissues. In the liver, it would be expected to have the opposite effect on ion balance that has been observed on the uptake and metabolism of acetate. While in acetate utilizing tissues, such as heart, changes in inorganic ion balance would be expected to be similar to the acetate treated liver. Thus, the effect of ethanol metabolism on the inorganic ion balance of liver, blood and other tissues will be examined.

Publications:

Gitomer, W.L. and Veech, R.L.: The accumulation of pyrophosphate by rat hepatocytes. Toxicol Ind Health 2: 299-307, 1986.

Gitomer, W.L. and Veech, R.L.: Application of near-equilibrium thermodynamics to living systems and the estimation of free mitochondrial  $\text{Ca}^{2+}$  concentration. In Lemasters, J.L., Hackenbrock, C.R., Thurman, R.G. and Westerhoff, H.V. (Eds.): Integration of Mitochondrial Function. New York, Plenum Publishing Corporation, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 AA 00023-09 LMMB
PERIOD COVERED <u>October 1, 1986 to September 30, 1987</u>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Effects of Ethanol on Metabolic Control Processes</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	R.L. Veech	Acting Chief
		LMMB, NIAAA
Others:	W.L. Gitomer	Senior Staff Fellow
		LMMB, NIAAA
COOPERATING UNITS (if any)  LCBG, NIDDK (R. Ornberg)		
LAB/BRANCH <u>Laboratory of Metabolism and Molecular Biology</u>		
SECTION <u>Metabolic Control</u>		
INSTITUTE AND LOCATION <u>NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852</u>		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Ethanol is metabolized in the liver by alcohol dehydrogenase to acetaldehyde, and acetaldehyde is then further metabolized by aldehyde dehydrogenase to acetate. Most emphasis in the study of the metabolites of ethanol has focused on acetaldehyde. However, the blood concentration of this compound rarely exceeds 10 uM, while the blood concentration of acetate approaches 2 mM even after the consumption of moderate amounts of ethanol. This study was undertaken to elucidate the consequences of acetate metabolism on the major metabolic pathways.</p> <p>Administration of acetate to starved rats causes a number of changes in hepatic metabolite concentrations, the most striking of which are (1) a greater than one hundredfold elevation in pyrophosphate concentration, (2) a fivefold elevation in calcium, (3) a marked reduction in the cytosolic [NADP+]/[NADPH] redox ratio, and (4) a twofold increase in glucose concentration.</p>		

Project Description:Investigators:

R.L. Veech  
W.L. Gitomer  
R. Ornberg

Acting Chief  
Senior Staff Fellow

LMMB, NIAAA  
LMMB, NIAAA  
LCBG, NIDDK

Objectives:

Most emphasis in the research concerning alcohol metabolism has centered on the possible reactions and potential significance of acetaldehyde. While of some theoretical interest, the thermodynamic characteristics of the reactions involved force the acetaldehyde concentration to remain very low, of the order of 2  $\mu$ M. In contrast, blood levels of 2 mM acetate are routine after drinking even very small quantities of ethanol since acetate is the primary product of ethanol metabolism. So far there has been very little systematic study of the metabolic effects of acetate. This study was therefore begun to elucidate the consequences of acetate metabolism on the major metabolic pathways, particularly its effect on the coenzymes and the PPI-related cofactor couples.

Methods Employed:

The study of the various pathways involved has required a whole series of new analytical methods for use in animal tissue. These methods are:

- (1) an enzymatic method for the spectrophotometric measurement of ribulose 5-P and ribose 5-P.
- (2) an HPLC method for measurement of purine and pyrimidine nucleotides.
- (3) an HPLC method for measurement of purine and pyrimidine bases.
- (4) an enzymatic method for measurement of phosphoribosylpyrophosphate.
- (5) an HPLC method for measurement of all the coenzyme A-derived nucleotides.
- (6) a carbon furnace atomic absorption method for measurement of Mg and Ca.
- (7) a surface coil method for obtaining NMR signal from PPI in rat liver in vivo in collaboration with the National Heart Institute.
- (8) a survey of localization of PPI and Ca using electron x-ray microprobe analysis.
- (9) a method for measurement of mitochondrial contents of PPI and Ca in isolated rat hepatocytes.
- (10) an enzymatic method for measurement of inorganic PPI.
- (11) digitonin fractionation of hepatocytes.

Eight of these eleven methods were developed in this laboratory.

Major Findings:

The administration of acetate to starved rats causes (1) a greater than one hundredfold elevation in pyrophosphate concentration, (2) a fivefold elevation in calcium, (3) a marked reduction in the cytosolic  $[NADP^+]/[NADPH]$  redox ratio, and (4) a twofold increase in glucose concentration. The Ca and PPi accumulate in the mitochondria as an insoluble salt.

In the area of methods development, this project has high significance. Having previously developed the first practical method to measure inorganic PPi in tissue or body fluids, we have now developed: (1) HPLC methods for measurement of most of the tri, di, and monophosphates, (2) an HPLC method for measurement of purine and pyrimidine bases, and (3) an HPLC method for measurement of the majority of tissue coenzyme A derivatives. These methods already are being used in a number of other research areas. Electron microprobe analysis showed a 10 fold increase in calcium accumulation in liver mitochondria after treatment with short chain fatty acids. Attempts are now being made to calculate free mitochondrial  $[Ca^{2+}]$ .

Significance to Biomedical Research and the Program of the Institute:

The elevation of PPi to 2 to 4 mM levels by acetate raises fundamental questions in a number of areas of metabolism. The elevation of liver and hence blood glucose by the cytoplasmic generation of PPi suggests the importance of this cofactor not only as a regulator of glucose, but as a previously unconsidered factor in diabetes.

Perhaps even more fundamental is the effect that PPi is known to have on DNA and RNA metabolism. Inorganic PPi at 2 mM levels is known to reverse DNA polymerase, with potentially marked effects on the genetics of the cells involved. In that regard, butyrate has long been known to detransform malignant cells, that is, to make these cells appear to behave as normal nonmalignant tissue. While the roles of acetylation of nuclear histones has heretofore been accepted as a partial explanation of this phenomenon, the role of PPi in detransformation will now have to be considered.

Elevation of intracellular PPi exerts the largest and most rapid changes in cellular calcium contents yet observed. Intracellular  $Ca^{2+}$ , along with cyclic-AMP, is the major cellular regulator of a multiplicity of reactions, from hormone actions of all sorts to malignant transformation by oncovirus. The role of PPi in controlling cellular  $Ca^{2+}$  will now have to be considered.

Finally, a number of disease states found in alcoholics have never been looked at from the point of view of the effects of acetate per se. Particularly obvious is the association of gout with alcohol intake in those patients with a genetic susceptibility to hyperuricemia.

Proposed Course:

The effects of changes in mitochondrial calcium on cellular energetics resulting from acetate are now being evaluated.

Publications:

Gitomer, W.L. and Veech, R.L.: The accumulation of pyrophosphate by rat hepatocytes. Toxicol Indust Health 2: 299-307, 1986.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00024-09 LMMB

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic and Metabolic Studies of Human Alcoholics

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory and institute affiliation)

P.I.: R.L. Veech Acting Chief LMMB, NIAAA

Others: J.P. Casazza Chemist LMMB, NIAAA

## COOPERATING UNITS (if any)

Department of Academic Medicine, London, England

## LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

## SECTION

Metabolic Control

## INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

0.6

## OTHER:

0.6

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

In three separate studies involving three different sets of collaborators, elevated levels of 2,3-butanediol have been found in the blood of 80% of chronic alcoholics, but not social drinkers consuming distilled spirits. Two separate methods of gas chromatographic analysis of diols have been developed. One method involving formation of the bromophenylboronate derivative can accurately measure to d-1, or meso 2,3-butanediol to 5  $\mu$ M.

In the rat, two pathways of butanediol formation have been demonstrated. The first (Veech et al.: Curr Top Cell Regul 18: 151-179, 1981) involves elevated blood acetaldehyde entering the brain with an active pyruvate dehydrogenase multi-enzyme complex where it condenses with hydroxyethyl thiamine pyrophosphate to form acetoin. The acetoin is subsequently converted in liver to 2,3-butanediol. In a second animal model, 2,3-butanediol in the rat is produced by acetone feeding. Prolonged fasting in man, however, produces diols but not 2,3-butanediol, suggesting differences in the metabolic pathways between man and the rat. Whether 2,3-butanediol in blood may be used as a "genetic marker" for alcoholism or is an induced defect can only be determined by a controlled study involving alcohol administration under controlled conditions on a metabolic ward.

Project Description:Investigators:

R.L. Veech	Acting Chief	LMMB, NIAAA
J.P. Casazza	Chemist	LMMB, NIAAA
E.D. Teague	Biologist	LMMB, NIAAA
M. Morgan	Professor	Dept. Academic Medicine, London, England

Objectives:

The purpose of this series of studies was to determine if alcoholics metabolize alcohol by a different pathway with different pathway controls or with different metabolic consequences than do nonalcoholics. The hypothesis to be tested thus derives from the classical medical paradigm and seeks to define a certain subset of people with alcohol problems who may be classified as being addicted to alcohol or are alcoholics (Rutstein, D., and Veech, R.L.: N. Engl. J. Med. 298: 1140-1141, 1978). The hypothesis to be tested, therefore, differs from the view that alcoholism is the result of deviant behavior patterns rooted in the sociocultural patterns of alcohol consumption and other forms of substance abuse.

Once abnormal or unusual metabolic consequences were identified within the alcoholic population, it followed that (1) accurate methods for measuring these compounds needed to be developed, and (2) the aberrant enzyme or gene product responsible for the metabolic difference needed identification. With accomplishment of these goals it would be possible, given an appropriate setting, to achieve a rational biochemical basis for the diagnosis of alcohol addiction by objective laboratory means and to determine whether these findings are a cause or a result of the alcoholism.

Methods Employed:

Identification of the abnormal metabolites was accomplished on contract (Borrison and JTC) using GC mass spectrometry. Measurement of diol was accomplished by use of a Varian gas chromatograph with electron capture detection. Acetol was measured as the dinitrophenylhydrazone derivative by HPLC. Synthesis of chemical intermediates of the diol pathway was accomplished by standard techniques of organic chemistry.

Major Findings:

**Clinical Studies:** Previous studies have shown blood 2,3-butanediol in 70% of blood samples and blood 1,2-propanediol in 90% of all samples drawn from alcoholics without liver disease when intoxicated (Rutstein, D.D., Veech, R.L., Nickerson, R.J., Felver, M.E., Vernon, A.A., Needham, L.L., Kishore, P., and Thacker, S.B.: Lancet ii: 534-536, 1983.) In alcoholics without liver disease as serum ethanol decreased so did the level of 2,3-butanediol. In most cases 2,3-butanediol was not measurable 32 hours after admission to a detoxification center (Veech, R.L., Felver, M.E., Lakshmanan, M.R., Huang, M.-T., and Wolf, S.: in Estabrook, R. and Srere, P. (Eds.): Current topic in cellular regulation. New York, Academic Press, 1981, Vol. 17, pp. 151-179). No 2,3-butanediol was measurable in control subjects in the absence of ethanol. In a more recent study (Casazza, J.P., Stambuk, D., Frietas, J., Morgan, M.Y. and Veech, R.L.: manuscript in preparation), ethanol-free blood samples were drawn from 53 normal reference subjects, 45



patients with alcoholic fatty liver, 10 patients with alcoholic hepatitis, 50 patients with alcoholic cirrhosis and 77 patients with non-alcoholic liver disease. 1,2-Propanediol was found in 8% of the reference samples, 15% of the patients with alcoholic fatty livers, 40% of the patients with alcoholic hepatitis, 46% of the patients with alcoholic cirrhosis and 49% of the patients with non-alcoholic liver disease. These data indicate that in the absence of ethanol, 1,2-propanediol is an indicator of general liver disease. Furthermore, administration of ethanol to controls and patients with non-alcoholic liver disease with measurable 1,2-propanediol resulted in a significant increase in 1,2-propanediol levels. Based on these data, it seems likely that the high incidence of 1,2-propanediol, previously found in blood drawn from alcoholics who were drinking distilled spirits (Rutstein *et al.*: Lancet ii, 534-536, 1983), was the result of ethanol raising 1,2-propanediol levels in a population capable of producing 1,2-propanediol due to pre-existing liver disease.

D,L-2,3-Butanediol was a more specific marker of alcoholic disease. D,L-2,3-butanediol was present in ethanol-free blood samples from 2% of the normal reference group, 9% of the patients with alcoholic fatty liver, 26% of the patients with alcoholic cirrhosis and 3% of the patients with non-alcoholic liver disease. The incidence of this compound within each group was independent of commonly used blood tests for liver disease. Administration of distilled spirits to 32 control subjects and 10 patients with non-alcoholic liver disease under controlled conditions did not result in any measurable D,L-2,3-butanediol. There was no difference in the incidence of D,L-2,3-butanediol in patients with alcoholic cirrhosis between those that had been ethanol abstinent two weeks or more and those that had not. In fact, the highest level of D,L-2,3-butanediol was identified in a patient who had been ethanol abstinent for 5 years. Clearly, despite the association of D,L-2,3-butanediol with alcoholism, the production of D,L-2,3-butanediol in these patients is not directly related to either ethanol metabolism or its metabolic products.

#### Metabolic Studies:

An ethanol inducible pathway for the production of 1,2-propanediol has been described in rat (Casazza, J.P., Felver, M.E., and Veech, R.L.: J Biol Chem 259: 231-236, 1984). The major inducible enzyme in this pathway has been shown to be P-450 2e, the major ethanol inducible enzyme (Koop, D.E. and Casazza, J.P.: J Biol Chem 260: 13607-13612, 1985). This enzyme has now been cloned (Song, B.J., Gelboin, H.V., Park, S.S., Yang, C.S., and Gonzalez, F.J.: J Biol Chem 261: 16687-16697). It has been suggested that P-450 2e plays a role in human oncogenesis (Thomas, P.E., Bandiera, S., Maines, S.L., Ryan, D.E. and Levin, W.: Biochemistry 26: 2280-2289, 1987). Clinical evidence of 1,2-propanediol production now indicates that this enzyme may play a role in liver disease.

#### Significance to Biomedical Research and the Program of the Institute:

The specificity of D,L-2,3-butanediol to alcoholic disease makes this compound of particular interest to alcohol research. The data to date are still compatible with but not conclusive of the possibility that the enzymes responsible for the elevation of D,L-2,3-butanediol represent an inherited metabolic abnormality distinguishing alcoholics from nonalcoholics. Even if D,L-2,3-butanediol is not a genetic indicator for alcoholism, a metabolic indicator of excessive alcohol consumption would be invaluable as a diagnostic tool for alcoholism. The close correlation between the incidence of 1,2-propanediol and liver disease suggests that understanding the mechanism of production of this compound may help explain the causes of liver disease.

Proposed Course:

Work in the Laboratory of Metabolism and Molecular Biology has identified D,L-2,3-butanediol as a specific indicator of alcoholic disease in both the absence of and the presence of ethanol. New animal models have been developed which result in the production of D,L-2,3-butanediol. In collaboration with the Royal Free Hospital, further studies into the production of 1,2-propanediol, meso-2,3-butanediol and D,L-2,3-butanediol are currently in progress. Known diol producers are being given postulated metabolic precursors in an attempt to further define the means of production of these compounds in humans. Work in this laboratory has led to the identification of 1,2-propanediol as a marker of liver disease, the description of a pathway for its production, and the cloning of the primary inducible enzyme of this pathway. Both the analytical methods developed for metabolic analysis and the techniques of molecular biology which have been developed are now being used to assess the role of 1,2-propanediol production in liver disease. Further studies into the mechanism of production of this compound in humans and the role of genetics in the production of these compounds, using the analytical techniques developed for metabolic analysis and the techniques of molecular biology, should give an insight into the etiology of alcoholism and liver disease.

Publications:

1. Casazza, J.P., Frietas, J., Stambuk, D., Morgan, M. and Veech, R.L.: The measurement of 1,2-propanediol, D,L-2,3-butanediol and meso-2,3-butanediol in controls and alcoholic cirrhotics. Alcohol Alcoholism, Suppl. 1:607-609, 1987.
2. Rutstein, D.D. and Veech, R.L.: Genetics and Alcoholism, Epidemiological Aspects in Genetics and Alcoholism. In Goedde, H.W. and Agarwal, D.P. (Eds.): New York, Alan R. Liss, Inc., 1987, pp. 33-44.
3. Veech, R.L., Gitomer, W.L. and Casazza, J.P.: Metabolic pathways leading to diol formation in Genetics and Alcoholism. In Goedde, H.W. and Agarwal, D.P. (Eds.): New York, Alan R. Liss, Inc., 1987, pp. 185-199.
4. Casazza, J.P., and Veech, R.L.: The production of 1,2-propanediol and 2,3-butanediol in severe alcoholics in Human Metabolism of Alcohol. In Crow, K (Ed): Boca Raton, CRC Press. "in press".

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 AA 00034-03 LMMB
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Control of the Level of Pentose Cycle Intermediates <u>In Vivo</u> .		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	J.P. Casazza	Chemist LMMB, NIAAA
Others:	R.L. Veech W.T. Schaffer	Acting Chief Executive Secretary LMMB, NIAAA OFA, NIAAA
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Metabolism and Molecular Biology		
SECTION Molecular Genetics		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  Of the pathological conditions associated with alcoholism, the etiology of Wernicke-Korsakoff's syndrome is one of the most well defined. Thiamine deficiency is clearly established as the causal factor. The pentose cycle, which supplies NADPH for the maintenance of cellular integrity and for fatty acid synthesis, is dependent on transketolase, a thiamine-dependent enzyme, for the functioning of this pathway. Despite the importance of the pentose cycle, neither control of flux nor of the level of pentose cycle intermediates is well understood. We have shown that in both starved and <u>ad libitum</u> fed animals the level of pentose cycle metabolites and metabolites of glycolysis are interdependent. The equilibrium relationships defined by the enzymes of the nonoxidative pentose cycle result in considerable stability in the level of pentose cycle intermediates. In dietary situations where the relative amounts of transketolase and transaldolase decrease with respect to glucose 6-P dehydrogenase, these equilibrium relationships are not valid. Under these circumstances, the tissue content of pentose cycle intermediates are elevated more than twenty-fold above those observed in starved animals.		

Project Description:Investigators:

J.P. Casazza	Chemist	LMMB, NIAAA
R.L. Veech	Acting Chief	LMMB, NIAAA
W.T. Schaffer	Executive Secretary	OFA, NIAAA

Objectives:

The purpose of this study was to determine if under any conditions the tissue content of glycolytic and pentose cycle intermediates were interdependent. It has been proposed that flux through glycolysis and the level of nucleotide synthesis is dependent on the level of 6-phosphogluconate (Smith, S.B., and Freedland, R.A.: *J. Biol. Chem.* 254: 10644-10648, 1980; Sommercorn, J., Steward, T., and Freedland, R.A.: *Arch. Biochem. Biophys.* 232: 579-584, 1984) and ribose 5-P (Boss, G.R.: *J. Biol. Chem.* 259: 2936-2941, 1984). The derived equilibrium relationships for each of the pentose cycle intermediates are functions of fractional powers of fructose 6-P and glyceraldehyde 3-P. These expressions indicate that large changes are required in glycolytic intermediates in order to effect relatively small changes in pentose cycle intermediates. Therefore, if equilibrium is maintained, the level of pentose cycle intermediates should not change significantly despite relatively large changes in the level of glycolytic intermediates.

Methods Employed:

Equilibrium determinations for each of the nonoxidative reactions of the pentose cycle and liver preparation were accomplished by standard laboratory techniques. Spectral assays for ribulose 5-P, xylulose 5-P, erythrose 4-P, sedoheptulose 7-P, and combined sedoheptulose 7-P and ribose 5-P were devised, as were improved enzyme assays for transketolase, transaldolase, and ribulose 5-P 3-carboxykinase.

Major Findings:

In ad libitum fed animals the levels of pentose cycle intermediates were described by both the tissue content of fructose 6-P and glyceraldehyde 3-P and the equilibrium constants for the nonoxidative enzymes of the pentose cycle under a number of conditions. The same was found for starved animals. In animals meal-fed a low-fat diet, these relationships were not found to be valid. An apparent disequilibrium in at least one of the major transketolase reactions resulted in large increases in the level of 6-phosphogluconate, xylulose 5-P, ribulose 5-P, and ribose 5-P.

In a related study, dehydroepiandrosterone (DHEA), an inhibitor of glucose 6-P dehydrogenase attributed with anticancer, antiobesity, and antiaging effects, was shown to have no effect on the  $[NADP^+]/[NADPH]$  ratio nor did it decrease the level of any of the pentose cycle intermediates measured. It has been reported that brain DHEA levels decrease one hundredfold after acute ethanol administration (Shoemaker, W.J., Corpechot, C., Bloom, F.E., and Baulieu, E.E.: *Alcoholism* 8: 119, 1984).

Significance to Biomedical Research and the Program of the Institute:

The changes observed in the level of pentose cycle intermediates in vivo represent some of the largest changes yet observed in a biological pathway. Whether these changes reflect some as yet undefined control mechanism for glucose 6-P dehydrogenase and what the effects of these changes are on other biological pathways are not clear.

Proposed Course:

The primary manifestations of Wernicke-Korsakoff's syndrome are neurological. Studies that will examine the effect of thiamine deficiency on pentose cycle intermediates in the brain are in progress.

Publications:

- I. Casazza, J.P., Schaffer W.T. and Veech R.L.: Letter to the Editor. J Nutr 117: 407, 1987.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 AA 00019-09 LMMB
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Pyrazoles as Affectors of Alcohol Dehydrogenase and Cytochrome P-450.</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	N.W. Cornell	Research Chemist
		LMMB, NIAAA
Others:	L.J. Marden	Staff Fellow
		LMMB, NIAAA
COOPERATING UNITS (if any)  Biochemistry Department, Dartmouth University, Hanover, NH (J. Sinclair). Biochemistry Department, Michigan Medical School, Ann Arbor, MI (D. Koop).		
LAB/BRANCH Laboratory of Metabolism and Molecular Biology		
SECTION Molecular Genetics		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.50	1.25	0.25
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>           All of these studies involve quantitative structure-activity analyses of pyrazole derivatives as probes of enzyme catalytic sites <u>in vitro</u> and as effectors of those enzymes within whole cells. This work began as an attempt to learn whether the properties of alcohol dehydrogenase determined with liver extracts have predictive value for that enzyme functioning in ethanol metabolism <u>in vivo</u>. The conclusions of that initial study had implications not only about alcohol dehydrogenase but also about the relative unimportance of other pathways of ethanol oxidation. However, those conclusions were potentially compromised by the demonstration in another laboratory that pyrazoles do, in contrast to long-standing assertions, inhibit microsomal ethanol oxidation. Thus, it became necessary to determine the structure-activity relationship for pyrazoles acting as ligands for cytochrome P450. That determination, in turn, suggested experiments with pyrazoles as inducers of P450 and the results of these experiments have provoked a working hypothesis about the unexplained mechanism of induction of some types of P450.         </p>		

Project Description:Investigators:

N.W. Cornell	Research Chemist	LMMB, NIAAA
L. Marden	Staff Fellow	LMMB, NIAAA
J. Sinclair	Associate Professor	Dartmouth University
D. Koop	Assistant Professor	University of Michigan

Objectives:

The long-range objective of this project is, with the use of a series of chemically defined pyrazoles, to elucidate the control of ethanol metabolism and the synthesis of ethanol-oxidizing enzymes.

Methods Employed:

The first phase of the work involved a quantitative structure-activity analysis for pyrazoles acting as inhibitors of alcohol dehydrogenase in vitro and of ethanol metabolism by intact cells. The second phase involves a similar analysis for the induction by pyrazoles of cytochromes P-450 in cultured hepatocytes. The same pyrazoles tested as inducers have also been characterized with regard to their ability to inhibit mixed function oxidation by microsomes in vitro. Most recently, a structure-activity analysis has been conducted for pyrazoles as ligands for cytochrome(s) P<sub>450</sub>. This involves obtaining different spectra for P<sub>450</sub>, either in purified form or in isolated microsomes, in the absence and presence of pyrazoles. These type 2 binding spectra give maxima at about 430 nm and minima at about 390 nm, and, by titration the association constants can be calculated using absorption-saturation relationships.

Major Findings:

**Alcohol dehydrogenase:** When pyrazoles were characterized as inhibitors of this enzyme in vitro and as inhibitors of ethanol metabolism by intact hepatocytes, the structure-activity relationships indicated (1) that the catalytic properties of alcohol dehydrogenase are similar in both environments and (2) that when pyrazoles inhibit ethanol metabolism in cells they do so by acting at a site chemically identical to the catalytic site of ADH. The latter was taken as indicating that the non-ADH pathways made no significant contribution to ethanol elimination under our conditions: this is discussed further in section B. The structure-activity relationships also showed a dependence on pyrazole hydrophobicity indicating that, when these compounds leave an aqueous medium to bind to the enzyme active site, the constraints are the same as when they partition between water and the reference solvent, octanol. In other words, the pyrazole must be completely desolvated in order to fit in the enzyme active site. That indication was confirmed in a computer graphics study based on the atomic coordinates of alcohol dehydrogenase. The pyrazole side chain was shown to lie in a narrow hydrophobic channel approaching the catalytic site and the heterocyclic ring is in a narrow slit between the side chains of a phenylalanine and a serine. From spectral and titration data, other workers had concluded that the N<sub>1</sub> nitrogen of pyrazole interacts with the positively charged pyridine ring of NAD, and the N<sub>2</sub> nitrogen bonds to the active site zinc atom. In agreement with that, computer graphics showed that both pyrazole nitrogens are within bonding distance, N<sub>2</sub> 2.0-2.2 Å from the catalytic zinc and N<sub>1</sub> 2.0 Å from the C<sub>4</sub> carbon of the nicotinamide moiety of NAD. These results support conclusions drawn from the structure-activity analysis. With the accumulation of such comparisons, there will come increased confidence in deductions about active site chemistry drawn from quantitative structure-activity studies.



**Pyrazoles as ligands for cytochrome P-450:** The report by Damgaard in 1982 showing that 4-methyl pyrazole is a competitive inhibitor of microsomal ethanol oxidation (a cytochrome P<sub>450</sub> reaction) raised questions about a conclusion mentioned above, namely, the one stating that ethanol metabolism was due almost entirely to alcohol dehydrogenase. This was based on the observation that inhibition of the enzyme in vitro and inhibition of metabolism by whole cells had identical dependences on the electronic character of the pyrazoles. Note that this does not say that pyrazoles act at a single, "specific" site; it does suggest that, even if they have multiple interactions, pyrazoles block cellular ethanol metabolism by acting at a site chemically like the catalytic center of ADH. However, the latter statement could not be made if the binding of pyrazoles to cytochrome P<sub>450</sub> happened to be defined by the same substituent parameters as with ADH. For that reason, structure-activity analyses were conducted with microsomes from rat and chick embryo liver and with the purified, ethanol-induced rabbit P<sub>450</sub>, LM3a. In all cases, dissociation constants were determined by spectrophotometric titration, and the difference spectra were type II (minimum at 390 nm, maximum at 425-430 nm) as is characteristic for amine ligands. For both sets of microsomes and the purified LM3a, the binding of pyrazoles was strongly dependent on hydrophobicity, but, in contrast to ADH, it was not affected by the electronic character of the substituents placed on pyrazole. Also, with LM3a, the structure-activity relationship for pyrazoles acting as inhibitors of p-nitrophenol hydroxylation was the same as for pyrazole binding. Thus, the action of pyrazoles on the microsomal mixed function oxidase system is governed by chemical properties that differentiate it from their action as inhibitors of alcohol dehydrogenase.

**Pyrazoles as inducers of cytochrome P-450:** Pyrazole (the parent compound) has been shown to induce small increases (30%) in total hepatic P<sub>450</sub> but about 4-fold increases in the dimethylnitrosamine demethylase activity. The latter is one of the catalytic potentials of the ethanol-induced isozyme of cytochrome P<sub>450</sub>, and it has also been shown that pyrazole induces this isozyme. Since the dissociation constants for 4-substituted pyrazoles and P<sub>450</sub> vary over a range of  $10^{-3}$ – $10^{-7}$  M, it was of interest to see whether any of these compounds might give greater induction of P<sub>450</sub> than that obtained with the parent molecule. That was tested with cultured chick embryo hepatocytes, a system chosen because, in contrast to cultured mammalian hepatocytes, it both retains the basal levels of P<sub>450</sub> and gives responses to a variety of xenobiotic inducers like those seen with adult chickens and mammals in vivo. The use of this culture system also obviates many of the difficulties associated with experiments in vivo and allows known concentrations of inducers to be tested at the site of interest, the liver cell. The quantitative structure-activity relationship obtained for induction of P<sub>450</sub> exactly parallels that for pyrazoles as ligands of P<sub>450</sub>; i.e., both relationships are linearly dependent on pyrazole hydrophobicity and no other substituent parameter. It is possible that this is coincidental. However, as a working hypothesis it is proposed that the similarity in the two relationships suggests that the ligands and the P<sub>450</sub>'s to which they bind act as co-inducers for the synthesis of those same P<sub>450</sub>'s. This proposed mechanism is analogous to that found for the DNA methyl transferase induction whereby the suicide inactivated enzyme promotes transcription of its gene. With regard to pyrazoles, our studies with microsomes from the marine fish, S. chrysops, which lacks barbiturate-type P<sub>450</sub>'s, showed that pentyl pyrazole binding was only 0.01% than that seen with chick embryo or rat liver microsomes. Pentyl pyrazole also failed to induce P<sub>450</sub> in S. chrysops when it was injected in vivo; this same compound gave a 7-fold induction of P<sub>450</sub> with the chick hepatocyte cultures. Thus, consistent with the proposal made above, pentyl pyrazole is a good inducer only in organisms possessing P<sub>450</sub>(s) that bind it with high affinity.

#### Significance to Biomedical Research and the Program of the Institute:

This work shows that the major factor determining the rate of ethanol elimination in vivo is the cellular activity of alcohol dehydrogenase. It confirms in a unique way that this enzyme behaves in the same way in the test tube as it does in its natural environment, the cell. The

results also provide, for the first time, some quantitative criteria for designing inhibitors to block ethanol and methanol toxicity in vivo. It also gives insight into the mechanism of induction of cytochromes P<sub>450</sub>, information that has not been forthcoming from other approaches to this problem.

Proposed Course:

This project will be completed in FY 1988.

Publications:

Cornell, N.W., Sinclair, J.F., Stegeman, J.J. and Hansch, C.: Pyrazoles as effectors of ethanol oxidizing enzymes and inducers of cytochrome P450. Alcohol and Alcoholism, Suppl. 1: 251-256, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00026-05 LMMB
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Subcellular Distribution of Enzymes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between; padding: 5px;"> <span>P.I.: N.W. Cornell</span> <span>Research Chemist</span> <span>LMMB, NIAAA</span> </div>		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Metabolism and Molecular Biology		
SECTION Molecular Genetics		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852		
TOTAL MAN-YEARS: 0.25	PROFESSIONAL: 0.25	OTHER: 0
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div> <input type="checkbox"/> (b) Human tissues         </div> <div> <input checked="" type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>           The metabolism of ethanol perturbs the major nucleotides of liver, the pyridine nucleotides (NAD, NADH), and the adenine nucleotides (ATP, ADP, AMP). Distributions of adenine nucleotides are also central to considerations of cellular bioenergetics, and accurate quantitative data for the subcellular distributions of enzymes are essential in analyzing metabolism <u>in vivo</u>. Compared with traditional techniques of tissue homogenization, digitonin fractionation of isolated hepatocytes yields a much faster and, in some cases, more accurate determination of enzyme compartmentation. Results with ATP citrate lyase are illustrative. Although previously thought to be entirely cytosolic, digitonin fractionation shows that a portion of total cellular ATP citrate lyase is bound to mitochondria or some other structure. The amount bound varies with the animal's nutritional state. In hepatocytes from rats that were either starved 2 days and then fed NIH stock diet <u>ad libitum</u>, or starved 2 days and then refed a fat-free diet 2 days, the noncytosolic activity was, respectively, 52%, 21%, or 24% of total cellular lyase. Because starvation/refeeding strongly induces lipogenic enzymes, the amount of bound lyase activity in this dietary state was 10 to 12 times greater than in rats that were starved or fed <u>ad libitum</u>. The association of citrate lyase with a subcellular organelle is also influenced by CoA. Adding 20 uM CoA to the digitonin fractionation medium caused all the lyase to be released from cells like a cytosolic enzyme. Conversely, when cellular free CoA was decreased by incubating hepatocytes with the hypolipidemic agent, 5-(tetradecyloxy)-2-furoic acid, the amount of bound lyase was increased. These results suggest that the noncytosolic ATP citrate lyase may have a special role in lipogenesis. Intracellular compartmentation and metabolic zonation can also be studied by perfusing the intact liver with digitonin, and calcium ions have a strong influence on relative patterns of enzyme release from the cytosol and mitochondria. This procedure has led to ways of isolating periportal and perivenous hepatocytes, enabling the study <u>in vitro</u> of metabolic zonation.         </p>		

Project Description:Investigators:

N.W. Cornell

Research Chemist

LMMB, NIAAA

Objective:

In current discussions, the term metabolic regulation generally implies phenomena such as allosteric inhibition or activation, enzyme interconversion, and induction or repression. Perhaps because it is so fundamental, it often is forgotten that enzyme compartmentation plays a major role in the integration and control of cellular metabolism. One example is the production within mitochondria of the carbamylphosphate used to initiate ureogenesis and the production in the cytosol of the carbamylphosphate for the biosynthesis of pyrimidines. Thus, having two CP synthetases located in different cellular compartments prevents the process of ammonia elimination from interfering with pyrimidine biosynthesis. Cellular compartmentation of proteins takes on added significance in view of recent discoveries of receptor recycling between the plasma membrane and the cell interior, and of maturation and translocation into organelles of proteins synthesized in the cytosol under control of the nuclear genome. These considerations make it desirable to be able to rapidly and accurately quantitate the subcellular distribution of enzymes and other macromolecules.

Methods Employed:

The problems outlined above have been studied in the present work with the use of digitonin, a nonionic, weak detergent that forms a tight complex with cholesterol. Digitonin had been found in the 1960's by Levy and Schnaitman and Greenawalt to be useful in selectively releasing enzymes from various submitochondrial compartments. Vignais in 1971 extended that observation to show that the rate at which digitonin would disrupt cellular membranes depends on the cholesterol content (e.g., plasma membrane; outer mitochondrial and microsomal membranes; inner mitochondrial membranes). An important application of those developments occurred in 1974 when Zuurendonk and Tager used digitonin to fractionate isolated hepatocytes in order to study metabolite compartmentation. The procedure described by those workers and applied by others required a 30- to 60-second fractionation period, and, as we believed those times to be long relative to many cellular processes, in 1980 we undertook a systematic evaluation of the digitonin fractionation of isolated rat hepatocytes.

Major Findings:

- (1) The establishment of methods that permit in 3 to 10 seconds the fractionation of rat hepatocytes into cytosolic and particulate materials;
- (2) Demonstration that the method provides a means of quantitating subcellular enzyme compartmentation that is much more rapid and accurate than traditional techniques of homogenization and differential centrifugation;
- (3) The discovery that, although 15 enzymes behave as predicted from results with classical techniques, three others (aspartate aminotransferase, malate dehydrogenase, and ATP citrate lyase) have quite unexpected distributions;
- (4) The discovery of a fraction of ATP citrate lyase that changes subcellular location in response to the metabolic state of the cell, which is phosphorylated in response to glucagon, and for which the degree of phosphorylation depends on the subcellular location.

The validity of the results listed in (3) above was established by electrophoresis showing that the cytosolic fractions obtained by digitonin treatment of hepatocytes contain none of the mitochondrial isozymes of aspartate aminotransferase or malate dehydrogenase or vice versa. Thus, the cytosolic: mitochondrial distributions of those enzymes are 22:78 for MDH as opposed to literature values of 70:30; for AspAT the corresponding values are 15:85 (digitonin) and 50:50 (literature). The digitonin procedure also indicates that adenylate kinase is located exclusively in the mitochondrial intermembrane space in contrast to published conclusions that this enzyme is 20% cytosolic.

Although digitonin fractionation has been extensively used to study metabolites, we have been less interested in that application because this or any other cell compartmentation fractionation procedure gives only total contents while the values needed are concentrations of free, metabolically active substrates. We have, however, studied this application to establish that, for obtaining valid measurements of adenine nucleotide compartmentation, the exposure to digitonin must not exceed 7 seconds with the fractionation medium at 1°C. These limitations are much narrower than those accepted by any of the groups that have reported metabolite compartmentation values obtained by the digitonin technique.

The digitonin fractionation procedure has recently been used to test conflicting reports about the subcellular distribution of rat liver phosphoenolpyruvate carboxykinase (PEPCK), an obligatory enzyme in the production of glucose from the major 3-carbon precursors, lactate and alanine. While many workers hold the view that gluconeogenesis in the rat proceeds via cytosolic PEPCK with the malate-aspartate shuttle being required to move oxaloacetate from mitochondria to the cytosol, there have been reports indicating that as much as 26-28% of liver PEPCK is mitochondrial, and those reports have been used to support the suggestion that mitochondrial PEPCK plays an important role in gluconeogenesis in the rat. If that were so and if the relative levels of cytosolic and mitochondrial PEPCK were to change with nutritional state as they do in other organisms. The current views of the control of rat hepatic gluconeogenesis would have to be modified substantially. However, the results obtained by digitonin fractionation of hepatocytes from rats that were either fed ad libitum or starved for 48 hours (which induces PEPCK) showed that 97% or more of the activity is located in the cytosol. The reports of substantial amounts of mitochondrial activity appear to be additional instances whereby the traditional techniques of cell fractionation can give erroneous estimates of enzyme distribution.

In addition, we now have shown that digitonin fractionation can be used to separate enzymes of the cytoplasm and lysosomal compartments. Since the lysosomal membrane has the second highest (after the plasma membrane) cholesterol content, studies on the aspect of enzyme compartmentation require much greater attention to the ratio of digitonin: cellular cholesterol. We have found that by keeping the molar ratio of digitonin: plasma membrane cholesterol at 0.75 - 1.0 it is possible to obtain 90-95% release of the cytoplasmic marker, LDH, with only 2% release of the lysosomal marker, acid phosphatase. This development provides the means to study the translocation of enzymes between cytoplasm and lysosomes as well as between cytoplasm and mitochondria.

The fractionations described above and all others we have performed were conducted with freshly isolated hepatocytes incubated in suspensions. As many studies are now being performed with hepatocytes cultured on solid supports, it was decided to see whether the digitonin fractionation procedure could be applied with cultured cells as well. The tests were run with cells attached to collagen plates and kept in culture for 48 hours, and these cells were surprisingly resistant to digitonin. At 37°C and digitonin concentrations of 1-4 mg/ml, 95% release of the cytoplasmic marker, lactate dehydrogenase, is obtained in 5-10 seconds. However, with the cultured cells and 16 mg of digitonin/ml, only about half of the total LDH was

released after 60 seconds exposure. This observation raised a question about which cell preparation had plasma membranes like those for parenchymal cells in vivo. To examine that question, it was decided to perfuse the whole liver with digitonin, thus, exposing hepatocytes to digitonin without the enzymatic treatment involved in cell isolation.

The results obtained with digitonin perfusion were quite unexpected. The liver is rapidly decolored, becoming almost white in about 1-2 minutes. Even more remarkable is the pattern of enzyme release. The rapid release of lactate dehydrogenase was similar to that obtained with freshly isolated cells, but when the releases of other cytoplasmic enzymes were expressed relative to LDH, it became clear that, by comparison to microdissection studies, that digitonin acts sequentially on cells located along the length of the sinusoid in the direction of perfusion. In other words, when the perfusion flow is from the hepatic portal vein to the vena cava, cell disruption begins with periportal hepatocytes and proceeds to the perivenous end of the sinusoid. When the perfusion direction is reversed, cells in the perivenous region are disrupted first. This picture of the perfusion pattern is supported by zymograms for pyruvate kinase. Liver contains two forms of that enzyme, the L-type which is located exclusively in parenchymal cells and an M-type that is only in the cells lining the sinusoid. By electrophoresis and antibody precipitation, it was found that the M-type is released over the same time course as lactate dehydrogenase. If cell disruption by digitonin proceeded radially from the entire length of the sinusoid, release of the M-type pyruvate kinase would be complete much sooner than the release of lactate dehydrogenase. With the digitonin perfusion it is possible to assess enzyme distribution along the microcirculatory unit of the liver, and the patterns obtained compare well with those from the much more lengthy and demanding technique of microdissection. This procedure has now been applied in other laboratories to isolate hepatocytes from each end of the sinusoid.

More recently, we have focused on improving the separation of mitochondrial and cytoplasmic enzymes obtained by digitonin perfusion. The aim here, as with isolated hepatocytes, is to obtain fractions containing cytoplasmic materials minimally contaminated with mitochondrial contents and vice versa. Early work with the digitonin perfusion showed a more rapid and extensive release of enzymes from the mitochondrial matrix than is seen when isolated cells are exposed to digitonin. Since the mitochondrial inner membrane is almost devoid of cholesterol, the observed releases of matrix enzymes appeared to be due to something other than the action of digitonin. This has now been traced to the presence of calcium in the Krebs/Henseleit medium used for the digitonin perfusions. Mitochondria are known to take up calcium when phosphate is also present in the medium (as it, also, is in Krebs/Henseleit saline), and as they concentrate calcium they swell and are lysed. When calcium-free Krebs/Henseleit is used for the perfusion medium, there is much lower release of matrix enzymes but undiminished release of cytoplasmic markers. For example, with 5 mg of digitonin/ml of perfusion medium about 80% of the total liver lactate dehydrogenase is released in 120 seconds both in the presence and in the absence of calcium. However, in that time period only 5% of the total citrate synthase is released when calcium is absent compared with 22% when calcium is present. It is expected that further manipulations of the perfusion medium and in the perfusion flow rate will result in a pattern that even more closely resembles that seen with isolated hepatocytes, nearly complete release of cytoplasmic markers with little or no release of mitochondrial matrix materials. This method should then be useful in studying the distribution of enzymes that are contained in the liver at levels too low to permit accurate studies with the small amounts of cellular material that can be processed in experiments with isolated hepatocytes.

Significance to Biomedical Research and the Program of the Institute:

Alcohol exerts its effects on hepatic metabolism by altering the redox state of the pyridine nucleotides, and a full understanding of those effects requires reliable quantitative information about enzymes such as malate dehydrogenase and aspartate aminotransferase that are setting the redox state, and enzymes like those of lipogenesis that respond to the redox state.

Proposed Course:

This project will continue in FY 1988.

Publications:

Cornell, N.W., Stegeman, J.J., Kerich, M.J., and Woodin, B.R.: Metabolite and Enzyme Contents of Freeze-Clamped Liver of the Marine Fish *Stenotomus Chrysops*. Comp. Biochem. Physiol. 85B: 669-674, 1986.





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00027-05 LMMB
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Induction of Aminolevulinic Acid Synthase in Hepatocytes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	N.W. Cornell	Research Chemist
		LMMB, NIAAA
Others:	R.H. Miller	Staff Fellow
		LMMB, NIAAA
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Metabolism and Molecular Biology		
SECTION Molecular Genetics		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The ingestion of alcohol causes porphyria in man and, with it, an increase in aminolevulinic acid synthase, the initial enzyme of heme biosynthesis. Since that enzyme is synthesized in the cytosol under the control of the nuclear genome, but must move from the cytosol into the mitochondria to function in heme biosynthesis, there are obviously many steps at which ethanol might act to increase its cellular activity. Previous studies in this laboratory have shown that the digitonin fractionation of isolated hepatocytes is an unusually good method for obtaining rapid information about the subcellular distribution of macromolecules, and this project was initiated to apply that method to analyze the induction of the synthase and its movement between cytosol and mitochondria. Evidence has been presented that, in chick embryo liver, the enzyme appears in the cytosol initially as a 74,000-molecular weight precursor that is converted to a 68,000-molecular weight protein during transit into the mitochondria. In contrast, for rat liver, it has been reported that both the cytosolic and mitochondrial ALAS proteins are dimers of 51,000-molecular weight subunits. These discrepancies may really reflect species differences, or they may simply arise from technical difficulties in extracting the native protein. Another contrast between the chick embryo and rat, mouse, guinea pig, as well as the adult chicken, is that all of the other named species can acquire substantial levels of ALAS in the cytosol following treatment with inducers (see below) whereas no cytosolic ALAS occurs in the chick embryo liver. These problems are being pursued by establishing conditions for the induction of aminolevulinic acid synthase in isolated liver cells and fractionation of these cells with digitonin. A cDNA probe is being developed to permit quantitation of ALAS mRNA levels in response to nutritional, alcohol and xenobiotic treatments.</p>		

Project Description:Investigators:

N.W. Cornell  
R.H. Miller

Research Chemist  
Staff Fellow

LMMB, NIAAA  
LMMB, NIAAA

Objectives:

The specific aims of this project are to establish conditions that permit the induction of aminolevulinic acid synthase to be studied in suspensions of isolated hepatocytes; to analyze the kinetics of messenger RNA transcription and translation and the movement of newly synthesized aminolevulinic acid synthase from cytosol to mitochondria; and to define the mechanism(s) by which alcohol and carbohydrates regulate the levels of the enzyme.

Methods Employed:

Hepatocytes are prepared from rat liver by the collagenase perfusion technique using a medium supplemented with a plasma amino acid mixture. Most amino acids are present at the concentrations characteristic of rat blood, but six of the essential amino acids shown to have strong activity in suppressing protein degradation are present at 4x plasma concentrations. The cells are also isolated in the presence of penicillin and streptomycin to permit their long-term retention in suspension without bacterial overgrowth. Cells from normal and induced animals are then subjected to digitonin fractionation, and the subcellular distribution of aminolevulinic acid synthase is measured relative to the marker enzymes, lactate dehydrogenase (cytosolic) and citrate synthase (mitochondrial).

Major Findings:

Rat liver aminolevulinic acid synthase has been difficult to work with because of its instability and its marked tendency to aggregate nonspecifically with other cellular proteins in homogenized liver. We have been able to stabilize the enzyme by keeping it constantly in the presence of 10% glycerol and a mixture of 5 different protease inhibitors. A combination of 1 mM dithioerythritol and 50 mM  $\text{MgSO}_4$  was found to release the enzyme from its aggregate and to cause it to run on Sephacryl S300 with an apparent molecular weight of 150,000-- the expected size from other studies with the chicken liver enzyme. When the enzyme is induced by treating rats for four hours with allylisopropylacetamide, it is distributed between cytoplasm and mitochondria in a 35:65 ratio. However, we have found that treating rats with hemin three hours after the inducer does not change the total enzyme seen at four hours, but it causes 100% of the activity to be located in the cytoplasm. This observation has two important implications: (1) heme appears to act first on the process of translocation and, perhaps, later or secondarily on transcriptional or translational events; (2) the turnover time for the mitochondrial enzyme is apparently much shorter than previously supposed. The literature gives half-lives of 45-120 minutes, but our results indicate a half-life of less than 20 minutes. Recently, we have shown that the enzyme is rapidly induced by ethanol, and the induction appears to be mediated by alcohol's profound decrease in succinyl CoA. One of the substrates for aminolevulinic acid synthase.

Significance to Biomedical Research and the Program of the Institute:

The general problem of protein movement between various subcellular compartments is relevant to many areas of biomedical research, and the approach to that problem described here should lead to more rapid and reliable measurements of protein movement than can be obtained by classical techniques. This work will also provide insight into the means by which alcohol consumption alters heme biosynthesis and metabolism.

Proposed Course:

This project will continue in FY 1988.

Publications:

King, M.T., Reiss, P.D. and Cornell, N.W.: Determination of short chain coenzyme-A compounds by reversed phase high-performance liquid chromatography. Meth. Enzymol., in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 AA 00033-04 LMMB
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Metabolic Effects of Growth Factors and Growth Hormone</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	B.Y. Reed	Senior Staff Fellow LMMB, NIAAA
Others:	M.J. Gerhart R.L. Veech	Chemist Acting Chief LMMB, NIAAA LMMB, NIAAA
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Metabolism and Molecular Biology		
SECTION Molecular Genetics		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             It has been demonstrated that chronic ethanol administration affects the ability of liver to regenerate <u>in vivo</u> by inhibiting <sup>3</sup>H thymidine incorporation into DNA following partial hepatectomy (McNeil, G.E., Chen, T.S. and Leevy, C.M.: <u>Hepatology</u> 5: 43-48, 1985; Poso, H., Vaananen, H., and Poso, R.: <u>Med Biol</u> 58: 329-336, 1980). More specifically ethanol has also been shown to inhibit hepatocyte DNA synthesis following hormonal stimulation by a number of agents including epidermal growth factor (EGF)(Carter, E.A. and Wands, J.R.: <u>Biochem Biophys Res Commun</u> 128: 767-774, 1985). In an attempt to understand the mechanism by which ethanol interferes with the normal processes of growth and development, we have undertaken an extensive study on the early metabolic changes induced by several hormones and growth factors <u>in vivo</u>. We have now identified the initial metabolite changes induced by platelet derived growth factor, epidermal growth factor and angiotensin in the rat <u>in vivo</u>. In addition, we have demonstrated a direct effect of ethanol on the normal metabolic action of EGF <u>in vivo</u> (Gerhart, M.J., Reed, B.Y. and Veech R.L.: submitted to <u>Alcoholism: Clin Exp Res</u>, 1987). Further studies are in progress to investigate the cause of the enzyme activity changes responsible for the observed metabolite changes <u>in vivo</u>.           </p>		

Project Description:Investigators:

B.Y. Reed	Senior Staff Fellow	LMMB, NIAAA
M.J. Gerhart	Chemist	LMMB, NIAAA
R.L. Veech	Acting Chief	LMMB, NIAAA

Objectives:

The aims of this project are to investigate the cellular mode of action of both GH and various growth factors and to assess the contribution made by GH or growth factor action to alcoholic liver damage and normal growth and development.

Methods Employed:

Rats are injected with an appropriate dose of the growth factor or hormone in question. The hormone treatment in the case of the ethanol studies is preceded by an acute dose of ethanol. The animals are sacrificed 5 min after administration of the hormone and metabolite levels and enzyme activities measured by established techniques on samples of their freeze clamped liver. All hormone treated animals are compared with similar data collected from saline injected controls.

Major Findings:

- (a) The initial intracellular metabolic changes induced by EGF in vivo have been demonstrated (Reed, B.Y., King, M.T. and Veech, R.L.: submitted to J. Biol. Chem., 1987).
- (b) EGF has been shown to have a direct effect on at least one enzyme in vivo and the responsible kinetic change has been elucidated.
- (c) Ethanol has been shown to modulate the normal metabolic actions of EGF in vivo (Gerhart, M.J., Reed, B.Y., and Veech, R.L.: submitted to Alcoholism: Clin Exp Res, 1987).
- (d) Platelet derived growth factor PDGF which has several similar effects to EGF, has been shown to act by a different mechanism to EGF in vivo. The metabolic mode of action of this growth factor has been identified (Reed, B.Y., King, M.T. and Veech, R.L.: J Biol Chem, 1987) in press.
- (e) PDGF has been shown to directly affect the Vmax activity of a key enzyme important for both the control of the flux of metabolites through the hexose monophosphate shunt and glycolysis.
- (f) EGF has been shown to increase intracellular free  $\text{Ca}^{2+}$ . The metabolic effects of EGF have been compared to those of another hormone, angiotensin, known to increase free intracellular  $\text{Ca}^{2+}$ . Both agents have been shown to differ in their metabolic effects in vivo.

Significance to Biomedical Research and the Program of the Institute:

Chronic alcohol consumption is known to affect the secretion of GH; whether alcohol has similar effects on the secretion or action of other growth factors is unclear. By investigating the basic mechanism by which GH or other growth factors act at a cellular level, new insight into the pathophysiology of some of the medical complications of alcoholism may be gained.

Proposed Course:

We are currently trying to identify the nature of the modification caused by EGF and PDGF to the enzymes affected by these agents. An understanding of this mechanism will allow a clearer insight into how ethanol modulates the normal action of growth factors in vivo. A cultured rat hepatocyte system is being developed to facilitate the study of the affected enzymes. As growth factors are known to play a critical role during growth and development, an attempt will be made to understand the long term effects of blocking normal growth factor action by ethanol.

The current studies are being expanded to an investigation of the growth hormone, somatomedin family of hormones, as ethanol is already known to have profound effects on the action of growth hormone in vivo.

Publications:

Reed, B.Y. and Veech, R.L.: The effects of chronic administration of T<sub>4</sub> growth hormone and epidermal growth factor on hepatic lipogenic enzymes in hypophysectomised rats. Biochim Biophys Res Commun 141: 78-83, 1986.

Reed, B.Y., King, M.T., Gitomer, W.L. and Veech, R.L.: Early metabolic effects of platelet derived growth factor and transforming growth factor beta in rat liver in vivo. J Biol Chem, 262: 8712-8715, 1987.

Gerhart, M.J., Reed, B.Y., Veech, R.L.: Ethanol inhibits some of the early effects of epidermal growth factor in vivo, Alcoholism (in press).





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00036-01 LMMB

## PERIOD COVERED

June 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and regulation of ethanol-inducible cytochrome P450 gene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: B.J. Song Senior Staff Fellow LMMB, NIAAA

Others: R.L. Veech Acting Chief LMMB, NIAAA

## COOPERATING UNITS (if any)

Laboratory of Molecular Carcinogenesis, National Cancer Institute (F.J. Gonzalez)

## LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

## SECTION

Molecular Genetics

## INSTITUTE AND LOCATION:

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The microsomal enzyme induced by feeding of alcohol is also the same enzyme responsible for the elevation of serum 1,2-propanediol found in the serum of alcoholics (Koop, D.R., Casazza, J.P.: J Biol Chem 260: 13607-13612, 1985). Specific polyclonal antibody against ethanol-inducible cytochrome P450 (P450j, P450 IIE) was prepared in rabbit and utilized to isolate cDNA for P450j. The cDNAs encoding P450j of both rat and human were isolated and characterized. The primary amino acid sequences of rat and human P450j were determined by nucleotide sequencing of the longest cDNAs for P450j. The rat P450j sequence was compared to those of other cytochrome P450s to determine the structural similarity.

Cloned P450j cDNA and antibodies were used to study the expression of P450j gene during development and by various inducers as well as pathophysiological conditions. By combination of cDNA hybridization and immunoblot analyses, three types of P450j gene expression were observed: transcriptional activation during development; post-transcriptional activation (probably via protein stabilization) by various inducers such as ethanol, acetone, and pyrazole derivatives; and mRNA stabilization in diabetes and starved animals. Distribution and regulation of P450j in different tissues were also studied by using cloned P450j cDNA.

Project Description:Investigators:

B.J. Song	Senior Staff Fellow	LMMB, NIAAA
R.L. Veech	Acting Chief	LMMB, NIAAA
F.J. Gonzalez	Acting Section Chief	LMC, NCI, NIH

Objectives:

The structure of ethanol-inducible cytochrome P450 (P450j) was to be determined by its cDNA nucleotide sequencing. Cloned P450j cDNA was used to study the basic mechanism of gene regulation by various inducers including ethanol, acetone and pyrazole derivatives which are known inhibitors of primary alcohol metabolizing enzyme, alcohol dehydrogenase. Its cDNA will also be used in human specimens for evaluating any possible polymorphism.

Methods Employed:

Identification of ethanol-inducible P450, P450j was performed by using specific antibody screening for both rat and human gtl1 cDNA libraries. Their respective nucleotide sequences were determined by dideoxy chain termination method. Elevation of enzyme activity of P450j (aniline hydroxylase) was compared to the level of expression of rat P450j during development and by various inducers by hybridization with P450j cDNA probe as well as immunoblot analysis. Elevation of P450j mRNA was further tested by nuclear transcription run-on assay to determine the nature of activation mechanism.

Major Findings:

- (1) The whole nucleotide sequences coding for ethanol-inducible P450 of rat and human (P450j) have been determined. Human P450j shared 75% nucleotide and 78% amino acid similarities to the homologous rat P450j cDNA and deduced protein.
- (2) Amino acid alignment also revealed that P450j was 48% similar to P450b and P450e, the major phenobarbital-inducible P450; 54% similar to P450PB1 and P450f, two developmentally regulated P450s (P450 IIC).
- (3) The induction of P450j by ethanol, acetone, and pyrazole derivatives was due to post-transcriptional activation without any increases in P450j mRNA level.
- (4) However, P450j gene is transcriptionally activated during development.
- (5) In chemically induced diabetic animals, the increases in P450j enzyme activity with concomitant increases in P450j protein was due to specific mRNA stabilization.
- (6) These three different types of regulation of P450j is rather unique in comparison to the regulatory mechanism of other P450s and appeared to be present in liver, lung, and kidney tissues.

Significance to Biomedical Research and the Program of the Institute:

Chronic intake of ethanol in both animal and human subjects leads to increases in the production of 1,2-propanediol, one of two unusual metabolites often found in human alcoholics blood (Rutstein, D.D. et al.: Lancet ii: 534-537, 1983). The P450j has been

demonstrated to be the responsible enzyme for the production of propanediol in experimental animals (Koop, D.R., Casazza, J.P.: J Biol Chem 260: 13607-13612, 1985). The works of basic protein biochemistry and future studies on human P450j from human specimens as analyzed by restriction fragment length polymorphism along with immunochemical analysis would give insights why and how people have different levels of propanediol after drinking alcohol.

#### Proposed Course:

Work will continue on elucidating the mechanism of P450j induction by ethanol or acetone. Studies using double radiolabelling of P450j with [ $^{14}\text{C}$ ]NaHCO<sub>3</sub> and [ $^3\text{H}$ ]leucine are in progress in an attempt to determine the rates of P450j protein turnover for both untreated control and acetone-treated animals.

#### Publications:

1. Song, B.J., Gelboin, H.V., Park, S.S., Yang, C.S. and Gonzalez, F.J.: Complementary DNA and protein sequences of ethanol-inducible rat and human P450s: Transcriptional and post-transcriptional regulation of the rat enzyme. J. Biol. Chem. 261: 16689-16697, 1986.
2. Song, B.J., Matsunaga, T., Hardwick, J.W., Veech, R.L., Yang, C.S., Gelboin, H.V. and Gonzalez, F.J.: Stabilization of cytochrome P450j mRNA in the diabetic rat. Mol. Endocrinol. in press.
3. Gonzalez, F.J., Kimura, S., Song, B.J., Pastewka, J., Gelboin, H.V. and Hardwick, J.P.: Sequence of two related P450 mRNAs transcriptionally increased during rat development. An Rdre 1 sequence occupies the complete 3' untranslated region of a liver mRNA. J Biol Chem 261: 10667-10672, 1986.
4. Gonzalez, F.J., Song, B.J. and Hardwick, J.P.: Pregnenolone 16 $\alpha$ -carbonitrile-inducible P450 gene family: gene conversion and differential regulation. Mol Cell Biol 6: 2969-2976, 1986.
5. Hardwick, J.P., Song, B.J., Huberman, E. and Gonzalez, F.J.: Isolation, complementary DNA sequence, and regulation of rat hepatic lauric acid w-hydroxylase (cytochrome P450<sub>LAw</sub>). Identification of a new cytochrome P450 gene family. J Biol Chem 262: 801-810, 1987.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 AA 00037-01 LMMB
PERIOD COVERED June 1, 1987 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular cloning of pyruvate dehydrogenase gene		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.	B.J. Song	Senior Staff Fellow LMMB, NIAAA
Others:	R.L. Veech	Acting Chief LMMB, NIAAA
	T.L. Huh	Visiting Fellow LMMB, NIAAA
	Y.T. Chi	Visiting Fellow LMMB, NIAAA
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Metabolism and Molecular Biology		
SECTION Molecular Genetics		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852		
TOTAL MAN-YEARS: 3.0	PROFESSIONAL: 3.0	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Recent studies from our laboratory indicate that 2,3-butanediol, one of two unusual metabolites found in human alcoholic blood, is predominantly associated with human subjects who suffer alcoholic hepatitis or alcoholic cirrhosis (Casazza <u>et al.</u>, <u>Alcohol and Alcoholism</u>, Suppl. 1: 607-609, 1987). The mechanism of the formation of 2,3-butanediol via pyruvate dehydrogenase was initially proposed by Veech <u>et al.</u> (Curr. Top. Cell. Regul. 18: 151-179, 1981). Based on the proposal, we have just started a project to clone pyruvate dehydrogenase (PDH) gene and to characterized its gene in light of developing a specific probe for a genetic marker for human alcoholism.</p>		

Project Description:Investigators:

B.J. Song	Senior Staff Fellow	LMMB, NIAAA
R.L. Veech	Acting Chief	LMMB, NIAAA
T.L. Huh	Visiting Fellow	LMMB, NIAAA
Y.T. Chi	Visiting Fellow	LMMB, NIAAA

Objectives:

The specific aims of this project are to study the structure and regulation of pyruvate dehydrogenase (PDH) gene at a molecular level and to develop a specific genetic probe for analysis of human specimens including human alcoholics.

Methods Employed:

Based on the published sequence of pyruvate dehydrogenase (Dahl et al., *J Biol Chem* 262: 7398-7403, 1987), several oligonucleotide probes were constructed and used to screen rat and human cDNA libraries. After identifying potential clones of PDH by hybridization their nucleotide sequences will be determined by dideoxy chain termination method. After confirmation of our clones as PDH cDNAs, they will be further used for the studies on PDH gene expression in the animal tissues from untreated and treated animals. Peripheral lymphocytes or skin fibroblasts obtained from human individuals including alcoholics and grown in tissue culture will be analyzed for human genetic polymorphism using cloned PDH cDNA probe with appropriate immunochemical approaches.

Major findings:

As this project was only initiated within a month, it is still in a developing stage. However, we are, so far, successful in identifying clones for pyruvate dehydrogenase E<sub>1</sub> $\alpha$  and E<sub>1</sub> $\beta$  subunits from rat brain cDNA libraries. The nucleotide sequences of these clones are being determined at this moment.

Significance to Biomedical Research and the Program of the Institute:

Based on the recent results and proposals from our laboratory, the presence of 2,3-butanediol is closely associated with those who had alcohol in the past and that it is generated from conjugation of acetaldehyde which is a metabolic product of ethanol with hydroxyethylthiamine pyrophosphate on the pyruvate dehydrogenase multienzyme complex. However, 2,3-butanediol can still be detected in those people who had not drunk for quite sometime, indicating that the production of this compound may not totally be dependent on the level of circulating acetaldehyde but rather depend on the genetic makeups of each individuals. The latter possibility will be carefully examined in an attempt to identify the molecular basis of individual differences in the level of 2,3-butanediol and to determine its potential as a genetic marker for human alcoholism as well as those who have problems of abnormal glucose metabolism such as patients of lactic acidosis with and without Leigh syndrome which is similar to Wernicke-Korsakoff encephalopathy in certain degree such as anatomical regions of affected brain areas. It is quite essential to the program of the Institute to study the research problems on human alcoholism by combination of molecular biology and immunochemical techniques.

Proposed Course:

After confirmation of our clones as genuine PDH  $E_{1\beta}$  and  $E_{1\alpha}$  subunits by nucleotide sequencing, these clones will be further used as specific probes for mRNAs from normal and alcoholic individuals. It has been demonstrated that defects in pyruvate dehydrogenase complex are inherited by autosomal recessive fashion as determined enzymatically. The possibility of genetic polymorphism will be also pursued by analyzing restriction fragment length polymorphism for genomic DNAs obtained from human subjects using cloned cDNA probes.

Publications:

None





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00438-08 LMMB
PERIOD COVERED October 1, 1986 to September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Protein Phosphorylation and Secretion and Ethanol Actions		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	H.C. Pant	Research Chemist LMMB, NIAAA
Others:	M. Virmani S.N. Ahmad	Research Chemist Visiting Fellow LMMB, NIAAA
COOPERATING UNITS (if any) Laboratory of Neurochemistry and Neuroimmunology, National Institute of Child Health and Human Development, NIH (H. Gainer). Laboratory of Neurobiology, NINCDS, NIH (P.E.Gallant).		
LAB/BRANCH Laboratory of Metabolism and Molecular Biology		
SECTION Physical Chemistry		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852		
TOTAL MAN-YEARS:	2.9	PROFESSIONAL: 2.4 OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>             The phosphorylation of protein has been proposed to be one of the molecular mechanisms involved in cellular regulation. The protein kinases catalyze these phosphorylation reactions. In this investigation, properties of protein kinase activity associated with cell cytoskeletal proteins have been studied in both neuronal and non-neuronal tissues. Extensive studies were carried out to characterize the squid axon neurofilament kinase. These investigations led to conclude that this kinase is a cyclic nucleotide- and calcium-independent protein kinase which is different from casein type I and type II kinases. The axoplasm and neurofilament preparation had no detectable protein kinase inhibitor activity, but strong inhibitor activity, which was not dialyzable but was heat inactivatable, was found in ganglion cells. This inhibitor activity may account for the low phosphorylation activity found in the stellate ganglion cells and may indicate inhibitory regulation of squid axon neurofilament kinase activity in the ganglion cell bodies. In rat brain, the kinase activity is associated with microtubule-associated proteins and phosphorylates them. Effects of ethanol on phosphorylation of microtubule associated protein (MAP 2) were investigated. Ethanol (4-24 mM) increased phosphorylation of MAP 2. In the presence of cAMP or mM ethanol, increased phosphorylation of MAP 2 was observed over control. Much higher phosphorylation of MAP 2 was observed in the presence of both cAMP and ethanol than the sum of phosphorylation of MAP 2 by cAMP and ethanol separately. Kinetic studies of the influence of ethanol on MAP 2 phosphorylation reveal an increased rate of phosphorylation of MAP 2 and a decreased Km in the presence of ethanol. These studies suggest that protein kinase(s) other than cAMP dependent protein kinase are influenced by ethanol and the enzyme(s) phosphorylate at distinct sites on MAP 2. The observation that ethanol affects MAP 2 provides an experimental basis for investigating the effects of ethanol on the structure and function of these cytoskeletal proteins and enzymes.           </p> <p>This project has been terminated.</p>		



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  <b>Z01 AA 00462-06 LMMB</b>
PERIOD COVERED <b>October 1, 1986 to September 30, 1987</b>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Ethanol and Membrane Function</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	H.C. Pant	Research Chemist LMMB, NIAAA
Others:	J. Shah	Visiting Fellow LMMB, NIAAA
COOPERATING UNITS (if any)  None		
LAB/BRANCH <b>Laboratory of Metabolism and Molecular Biology</b>		
SECTION <b>Physical Chemistry</b>		
INSTITUTE AND LOCATION <b>NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852</b>		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	0.7	0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             Ethanol is known to alter the biochemical and biophysical properties of cellular membranes. In this investigation the mechanisms of ethanol-induced membrane potential changes in brain synaptosomes and rat thymocytes were studied with fluorescence spectrophotometry using membrane potential-sensitive dyes. The effect of ethanol on membrane potential in synaptosomes was studied using rhodamine 6G fluorescence. The fluorescence signal responds to the depolarization produced by increasing concentration of K<sup>+</sup> outside the synaptosomes. The fluorescence intensity increases with increasing [K<sup>+</sup>]<sub>o</sub> from 5 mM to 60 mM and then levels off at higher concentrations. When the [Na<sup>+</sup>]<sub>o</sub> concentration was changed from 0 to 137 mM, keeping [K<sup>+</sup>]<sub>o</sub> constant at 5 mM, no change is observed in fluorescence. On addition of ethanol, the fluorescence intensity increased. There was a significant change (15%) in fluorescence intensity with as low as 10 mM ethanol. The fluorescence intensity increase as ethanol concentration was varied from 10 to 100 mM. Ethanol had no effect on depolarized (80 mM KCl) synaptosomes. The effect of ethanol on membrane depolarization was similar to K<sup>+</sup> depolarization. In the absence of synaptosomes ethanol had no effect in the fluorescence intensity of dye. In lysed or 12 hr. old synaptosomes, there was no change in fluorescence either with [K<sup>+</sup>]<sub>o</sub> or ethanol. We concluded that (1) Rhodamine 6G fluorescence can be used to monitor the change in membrane potential in synaptosomes (2) low doses of ethanol produces changes in membrane potential in synaptosomes isolated from rat brain.           </p> <p>This project has been terminated.</p>		



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER <b>Z01 AA 00474-04 LMMB</b>								
PERIOD COVERED <b>October 1, 1986 to September 30, 1987</b>										
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Ethanol and Nervous System Degeneration</b>										
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">P.I.:</td> <td style="width: 35%;">H.C. Pant</td> <td style="width: 35%;">Research Chemist</td> <td style="width: 15%;">LMMB, NIAAA</td> </tr> <tr> <td>Others:</td> <td>M. Virmani</td> <td>Research Chemist</td> <td>LMMB, NIAAA</td> </tr> </table>			P.I.:	H.C. Pant	Research Chemist	LMMB, NIAAA	Others:	M. Virmani	Research Chemist	LMMB, NIAAA
P.I.:	H.C. Pant	Research Chemist	LMMB, NIAAA							
Others:	M. Virmani	Research Chemist	LMMB, NIAAA							
COOPERATING UNITS (if any) Laboratory of Neurochemistry and Neuroimmunology National Institute of Child Health and Human Development, NIH (H. Gainer). Laboratory of Neurobiology, NINCDS, NIH (P.E.Gallant).										
LAB/BRANCH Laboratory of Metabolism and Molecular Biology										
SECTION Physical Chemistry										
INSTITUTE AND LOCATION NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852										
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:								
.60	.60									
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews										
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Brain damage occurs in over 60% of chronic alcoholics. The causes and pathophysiology of this damage, however, are poorly understood. The general goal of our research has been to evaluate the factors that lead to alcohol-induced degeneration of the nervous system in experimental animals. Ethanol has been shown to release calcium from intracellular storage sites (U. Pande and H.C. Pant, Neuroscience 9: 1235, 1983; J.B. Hoek and E. Rubin 13th International Congress of Biochem. 28, 1985). The increase in cytosolic calcium activates proteases cause proteolysis of cytoskeletal proteins. The possibility exist that some of the effects of alcohol-induced degeneration may be to the activation of these proteases. In order to investigate the role of intracellular calcium during neuronal degeneration we have used the squid giant axon as a model system. The advantages of using this preparation are that the enzyme and substrates can be extruded in high yield from the giant axon, thus avoiding contamination of the preparation with nonaxonal enzymes, which may become nonspecifically attached during the homogenization of whole tissue. Second, enzyme activities and substrates present in axoplasm and axoplasmic preparations can be compared with the activities and substrates present in the region of the stellate ganglion, which contains the cell bodies of the giant axon. In the squid axon and cell body we have identified a number of immunoreactive degradation products after calcium-activated proteolysis. The results suggest that an elevation of cytosolic ionized calcium may result in a breakdown of proteins.</p> <p>This project has been terminated.</p>										



Annual Report of the Laboratory for Physiologic and  
Pharmacologic Studies  
National Institute on Alcohol Abuse and Alcoholism  
October 1, 1986 - September 30, 1987  
Boris Tabakoff, PhD, Acting Chief

## Introduction

The Laboratory of Physiologic and Pharmacologic Studies (LPPS) was constituted in FY 86 and consists of the Section on Receptor Mechanisms, the Section of Electrophysiology, and the Section on Immunology. Both the Sections on Receptor Mechanisms and Electrophysiology have, during the current year, progressed in the development of their already established research themes. The progress within these sections is detailed below. The Section on Immunology is a newly established endeavor within the Laboratory and this section is beginning a research program focusing on ethanol's actions on the immune system and, as well, is studying the processes of communication between the brain and the immune systems of the body. Laboratory activities, as a whole, have been exemplary both in terms of research productivity and staff involvement in national and international meetings dealing with alcoholism. In addition, the Chief of the Section on Receptor Mechanisms has served as a member of an NIAAA Initial Review Group and as an organizer for the annual meeting of the Research Society on Alcoholism. The Chief of the Section of Electrophysiology has, in addition to his regular duties, managed the AIDS Research Program as the NIAAA AIDS Research Coordinator. The following sections describe the research activities of the Laboratory.

## Section on Receptor Mechanisms

The overall goal of the studies being performed within the Section on Receptor Mechanisms, Laboratory of Physiologic and Pharmacologic Studies, continues to be a determination of the neurochemical mechanisms responsible for neuroadaptive responses to ethanol (functional tolerance and physical dependence). Because neuroadaptation is believed to occur at the initial site of action of ethanol, our investigations also focus on the acute neurochemical effects of ethanol, as well as the mechanisms involved in acute behavioral responses to ethanol. Our research, and that of others, supports the postulate that neurotransmitter and neuromodulator receptors and receptor-effector coupling processes are sensitive to disruption by ethanol. Ethanol may alter the function of these systems either by perturbing the structure of neuronal membrane lipids, lipid-protein interactions, or the conformation of membrane-bound proteins. However, ethanol appears to have specific sites of action within these systems, and our investigations apply biochemical and molecular biological techniques to identify the initial response to ethanol and adaptations and/or pathological changes that occur following chronic ethanol exposure or ingestion. These changes, in addition to elucidating the mechanism of action of ethanol in the CNS, may prove useful as potential indicators of chronic alcohol consumption.

Another approach to elucidating the mechanisms underlying neuroadaptation to ethanol is to focus on neuronal systems and neuromodulators that influence various aspects of ethanol tolerance. We have previously

postulated the existence of intrinsic and extrinsic neuronal systems that affect tolerance. Extrinsic systems are those which modulate the acquisition, expression or dissipation of tolerance, while intrinsic systems are those in which tolerance to specific effects of ethanol is actually encoded, presumably by changes in synaptic efficacy. This framework has also been used to describe the neurobiology of learning and memory, processes which, like tolerance, represent adaptation of the CNS to external stimuli. The major focus of our recent work is the neurohypophyseal hormone, arginine vasopressin (AVP). This neuropeptide appears to represent an extrinsic system that modulates ethanol tolerance, and behavioral, biochemical and molecular biological techniques are being used to investigate the mechanism and sites of action of AVP in the CNS, as well as the effects of ethanol on regulation of AVP biosynthesis and release. Definition of the role of a naturally-occurring hormone in ethanol tolerance may lead to development of therapies to manipulate tolerance, and, as a consequence, alcohol drinking behavior.

1. Receptor-effector coupling processes and other membrane-bound proteins. The role of the GABA/benzodiazepine receptor-coupled chloride channel in the actions of ethanol was investigated with the benzodiazepine partial inverse agonist, Ro15-4513. This compound, reported to be an ethanol antagonist, was found to selectively antagonize certain behavioral effects of ethanol. The results provide insight into the mechanism of action of ethanol and further define the potential of the imidazobenzodiazepines as alcohol antagonists.

At the biochemical level, our studies indicate that acute and chronic ethanol exposure affect the function of Gs, the stimulatory guanine nucleotide binding protein. Changes in the function of this protein after chronic ethanol ingestion appear to significantly reduce the response to several agonists that increase adenylate cyclase activity, and could contribute to the disruptions in neuronal activity that occur in animals tolerant to and physically dependent on ethanol. These studies, as well as studies of (Na<sup>+</sup>,K<sup>+</sup>)ATPase, demonstrate the specificity of ethanol's effects, since ethanol seems to affect only Gs (not Gi or Go) and only one form (the neuronal form) of the enzyme. Differences in adenylate cyclase activity, as well as inhibition of monoamine oxidase activity by ethanol, have been successfully used, in an initial study of platelet enzyme activities, to distinguish alcoholic and non-alcoholic subjects.

Cell and organ culture systems have also been used to evaluate the effects of ethanol. While these systems are not influenced by physiological alterations that occur *in vivo*, and cannot be used to reflect behavioral aspects of adaptation, they have the advantage of relative homogeneity, the possibility to study physiological consequences of neurotransmitter receptor interactions, and the potential for producing large amounts of proteins of interest. Using PC12 cells, an inhibitory effect of ethanol on another receptor-effector coupling process, agonist-stimulated polyphosphoinositide metabolism, and consequent neurotransmitter release, has been observed. This system responds similarly to that in brain slices, but is more sensitive to ethanol, and may be used to provide a more detailed description of the effects of ethanol. In pineal glands in culture, the effects of ethanol on beta-adrenergic receptor-coupled melatonin production have been defined, and are similar to the effects of ethanol on beta-adrenergic receptor function in brain. Studies of the effects of ethanol on several receptor-coupled second



messenger systems in primary cultures of cerebellar granule cells, as well as on calcium metabolism in brain preparations, are also underway.

## 2. Extrinsic Systems that Modulate Ethanol Tolerance: Vasopressin.

By using agonists and antagonists that are selective for vasopressin V-1 and V-2 receptor subtypes, the brain receptors mediating the ability of vasopressin to maintain ethanol tolerance have been defined as V-1. Studies with antagonists have also demonstrated that endogenous vasopressin, interacting with V-1 type receptors, plays a role in the maintenance of ethanol tolerance. These findings pave the way for a determination of the neurochemical mechanism of action of AVP, and for the possible development of therapeutic agents that can be used to manipulate ethanol tolerance. Studies of the development of ethanol tolerance have shown that vasopressin, as well as agonists selective for either V-1 or V-2 receptors, oxytocin, and peptides related to oxytocin all delay or inhibit the acquisition of tolerance. This effect appears to involve an action of vasopressin different from that on maintenance of tolerance, and again indicates that benign and effective therapies for altering ethanol tolerance development may eventually be devised. Molecular biological studies have indicated that vasopressin is synthesized in extrahypothalamic tissues, including anterior pituitary and testis. Examination of the effects of ethanol on the regulation of hormone synthesis and release in hypothalamus, extrahypothalamic brain areas, anterior pituitary and in peripheral organs is underway to further clarify the role of endogenous AVP in mediating adaptation to ethanol.

## 3. Genetics and Adaptation to Alcohol

In order to more definitively ascertain the role of neurochemical systems in various aspects of ethanol tolerance and, possibly, physical dependence, breeding studies designed to produce selected lines of rats that develop high and low degrees of tolerance have been initiated. Theoretically, at the limit of selection, animals selected for each trait will possess all the alleles associated with that trait, while other alleles will be randomly distributed. These animals will be a resource for all researchers interested in the biochemical determinants of ethanol tolerance.

## Section on Immunology

The Section on Immunology is currently undertaking activities in two distinct areas. The first area relates to studies of ethanol's effects on cells of the immune system. In the completed studies mice and rats chronically fed ethanol-containing diets were found to have reduced numbers of lymphocytes both in peripheral blood and in the spleen and thymus. In addition, the ethanol-treated animals displayed a diminished ability of their lymphocytes to respond to proliferative stimuli. In general, ethanol compromised the ability of an animal to mount a primary immune response. In ascertaining the mechanism by which ethanol affects lymphocyte function, it was noted that interleukin 2 (IL-2) production was increased at the same time that cell proliferation was compromised. Current studies are focusing on possible ethanol-induced anomalies in IL-2 receptors and in signal transduction between the receptors and effector systems of lymphocytes.

Another line of investigation being pursued in the Section on Immunology is related to AIDS virus envelope protein action on brain tissue. The neurologic and cognitive deficits that accompany the progression of AIDS in many patients seem to be related to secondary effects of the AIDS virus, rather than to direct infection of neurons with the virus particles. A portion of the structure of the envelope protein gp120 exhibits structural homologies with vasoactive intestinal peptide (VIP). This portion of the molecule has been sequenced and synthesized and has been referred to as peptide T. Studies in our section on the physiologic effects of peptide T have demonstrated that intraventricular administration of this peptide produces a significant, anatomical-area specific enhancement in brain glucose utilization. The measurements of brain glucose utilization were carried out by quantitative autoradiography using radioactively labeled 2-deoxyglucose. Another peptide differing from peptide T by two amino acids was also used in these studies. This peptide is less effective than peptide T in binding to brain tissue receptors and thus has been assumed to be less potent in physiologic tests. High doses of this peptide, however, were found to also stimulate brain glucose metabolism and current work is focusing on the relative dose response functions of peptide T, as well as its substituted analog. These studies, as well, will involve examinations of VIP and VIP antagonist actions in brain to ascertain whether peptide T or AIDS envelope protein is interacting with VIP receptors in brain to produce the witnessed effects.

## Section of Electrophysiology

The research program of the Section of Electrophysiology is directed toward elucidating the cellular mechanisms involved in the acute and chronic actions of ethanol in the nervous system. Although the effects of ethanol on behavior are well known, the cellular mechanisms by which ethanol produces its effects on nervous system function are poorly understood. The investigations use modern electrophysiological, biophysical and optical methods to study the cellular and molecular mechanisms involved in ethanol's actions in nervous tissue. The research program of the Section encompasses investigations in two broad areas: nerve cell excitability and neurosecretory mechanisms. These two areas provide the organizational basis of this report.

### 1. Nerve Cell Excitability and Ion Channels.

Previous investigations have demonstrated that ethanol can alter the excitability of neurons in the central nervous system; however, the mechanisms involved in these effects are not known. In order to determine the alterations of ion channel function that underlie the effect of ethanol on nerve cell excitability, we have developed new experimental preparations (acutely isolated adult mammalian neurons) and applied recently developed biophysical methods (patch-clamp and single-electrode voltage-clamp recording) to investigate membrane ion channel function in mammalian neurons. We have identified and are characterizing the following ion currents in mammalian neurons: two sodium currents (tetrodotoxin-sensitive and tetrodotoxin-resistant), two calcium currents (transient and sustained), a calcium-activated chloride current, and three potassium currents (transient,

delayed rectifier and calcium-activated). These ion currents underlie action potential generation and steady-state membrane excitability in mammalian neurons. In order to determine how ethanol affects nerve cell excitability, we are investigating the effects of ethanol on these ion currents. These studies should increase our understanding of the mechanisms involved in ethanol's actions on nervous system excitability.

## 2. Neurotransmitter Release and Neurosecretion.

Previous studies have shown that ethanol can alter neurotransmitter release and neurosecretion, but the mechanisms involved in these effects have not been determined. It is well established that calcium ions play an essential role in the neurosecretory process. To investigate whether the effects of ethanol on neurosecretion result from alterations in cellular calcium metabolism, we are applying recently developed biophysical and optical methods. Using the patch-clamp recording technique, we are studying the cellular mechanisms that modulate membrane calcium current that triggers neurosecretion, and the effects of ethanol on those mechanisms. The studies indicate that the calcium channel can be regulated by a GTP-binding protein. In addition, our recent studies suggest that the calcium channel may also be regulated by protein kinase C. We are also studying the role of intracellular calcium ions in the neurosecretory process using optical methods with the fluorescent calcium-indicator, Quin 2, and have found a receptor mediated elevation of inositol triphosphate and intracellular calcium, and a release of neurotransmitter. Analysis of these phenomena indicates that the neurotransmitter release is associated with an inositol triphosphate-induced mobilization of intracellular calcium ions. Ethanol inhibits this receptor mediated release of neurotransmitter. Current experiments are directed toward elucidating the site of ethanol's action in this neurosecretory process by examining the effects of ethanol on the interrelationship between intracellular calcium mobilization, phosphoinositide metabolism and neurotransmitter release. The significance of this project derives from the fact that characterization of neurosecretory mechanisms and the actions of ethanol on those processes should increase our understanding of the mechanisms involved in ethanol's actions on neurotransmitter release and neurosecretion.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00479-04 LPPS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synaptic and Neurosecretory Mechanisms and Ethanol Actions

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	F. F. Weight	Section Chief	LPPS, NIAAA
Others:	L. G. Aguayo	Staff Fellow	LPPS, NIAAA
	S. J. Korn	Physiologist	LPPS, NIAAA
	C. S. Rabe	Senior Staff Fellow	LPPS, NIAAA

## COOPERATING UNITS (if any)

Lab. Cell Biol., NIMH, (A. Luini); Dept. Pharm., U. Alberta, (P.A. Smith); Dept. Physiol., Creighton U. (J.A. Wilson); Lab. Neurophysiol., NINCDS (D.L. Lewis); Marine Science Center, Univ. Oregon (P. Yavari)

## LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

## SECTION

Section on Electrophysiology

## INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

## TOTAL MAN-YEARS:

2.3

## PROFESSIONAL:

2.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous studies have shown that ethanol can alter neurotransmitter release and neurosecretion; however, the mechanisms involved in these effects have not been determined. We have studied neurosecretory mechanisms and the actions of ethanol on those mechanisms in several secretory cell types. In the ACTH secreting mouse pituitary cell line, AtT-20, we have studied both calcium current and calcium-activated chloride current. Calcium current in these cells is inhibited by somatostatin and analysis of the mechanism indicates that the inhibition involves a GTP-binding protein. Calcium current in these cells is also inhibited by protein kinase C activators. Analysis of the calcium-activated chloride current in AtT-20 cells reveals that the decay kinetics are largely due to mechanisms that regulate intracellular calcium. The relationship between intracellular calcium and neurosecretion was studied in the catecholamine secreting rat chromaffin cell line, PC12. In these cells, muscarine-stimulated release of catecholamine is associated with an inositol triphosphate-induced mobilization of intracellular calcium. Ethanol inhibits both the release of neurotransmitter and the increase of intracellular calcium. The membrane mechanisms associated with secretion are also being investigated in cells from the pineal gland of the adult rat. The significance of the project derives from the fact that characterization of neurosecretory mechanisms and the actions of ethanol on those mechanisms should increase our understanding of the cellular basis of ethanol's actions in the nervous and endocrine systems.

PROJECT DESCRIPTION:Investigators:

P.I.:	F. F. Weight	Section Chief	LPPS, NIAAA
	L. G. Aguayo	Staff Fellow	LPPS, NIAAA
	S. J. Korn	Physiologist	LPPS, NIAAA
	D. L. Lewis	Staff Fellow	LNP, NINCDS
	A. Luini	Visiting Fellow	LCB, NIMH
	C. S. Rabe	Senior Staff Fellow	LPPS, NIAAA
	P. A. Smith	Asst. Prof.	D.P., U. Alberta
	J. A. Wilson	Asst. Prof.	D.P., Creighton U.
	P. Yavari	Research Sci.	M.S.C., U. Oregon

Objectives:

Ethanol has been reported to alter synaptic transmission and neurosecretion in the nervous and endocrine systems; however, the mechanisms involved in these actions are poorly understood. The objectives of this project are to characterize synaptic and neurosecretory mechanisms and the actions of ethanol on those mechanisms.

It is well known that  $\text{Ca}^{2+}$  plays an important role in synaptic transmitter release and neurosecretion. Previous studies indicate that ethanol can alter  $\text{Ca}^{2+}$  metabolism in synaptosomes (pinched off nerve terminals). This suggests that the effects of ethanol on transmitter release and neurosecretion may result from alterations in cellular  $\text{Ca}^{2+}$  metabolism. Since it is not possible, at the present time, to study ion channels or intracellular  $\text{Ca}^{2+}$  signals in nerve terminals, we have conducted experiments on three types of neurosecretory cells: (1) the mouse pituitary cell line, AtT-20; (2) the rat chromaffin cell line, PC12; and (3) cells from the rat pineal gland.

Methods Employed:

## 1. AtT-20 cells:

## a. Calcium current:

Somatostatin inhibits the secretion of ACTH evoked by secretagogues including corticotropin releasing factor, vasoactive intestinal peptide, isoproterenol, and forskolin in the mouse pituitary cell line AtT-20 cells. In recent studies on AtT-20 cells, Luini et al. have found that: (i) somatostatin decreases the basal cytosolic  $\text{Ca}^{2+}$  rise evoked by the above secretagogues; (ii) nifedipine reduces basal and secretagogue stimulated cytosolic  $\text{Ca}^{2+}$  levels; (iii) the effects of somatostatin and nifedipine are not additive; and (iv) TEA does not block the effect of somatostatin on cytosolic  $\text{Ca}^{2+}$ . These results raise the possibility that somatostatin may affect membrane  $\text{Ca}^{2+}$  conductance. In view of this possibility, we used the patch-clamp method to study the effect of somatostatin on the  $\text{Ca}^{2+}$  currents in these cells.

AtT-20 cells were cultured in Dulbecco's Modified Eagle medium containing 10% fetal calf serum at 37°C in a humidified 10%  $\text{CO}_2$  atmosphere. After subculturing 8-10 days, membrane currents were recorded using the patch-clamp method in the whole cell voltage-clamp mode. The extracellular solution contained: 150mM TEA-Cl, 0.8mM  $\text{MgCl}_2$ , 5.4mM KCl, 10mM  $\text{CaCl}_2$ , 10mM HEPES/CsOH (pH 7.4) and 1μM tetrodotoxin with an osmolarity of 340 mosmol/kg. The intracellular patch

pipette solution contained: 120mM CsCl, 11mM EGTA, 2mM TEA-Cl, 2mM MgCl<sub>2</sub>, 10 HEPES/CsOH (pH 7.4), 4mM MgATP, 20mM creatine phosphate, and 50 U/ml creatine kinase with an osmolality of 318 mosmol/kg. The nonhydrolyzable GTP analog, guanosine 5'-(3-0-thio)triphosphate (GTP- $\gamma$ -S), was added to the patch pipette solution at a concentration of 100 $\mu$ M. Some patch pipettes had an additional 100 $\mu$ M cAMP and 1mM IBMX. Cells were voltage clamped to a holding potential of -80mV and stepped to +10mV, the peak of the current-voltage relationship for calcium current. All recording was at room temperature, 20-22°C. Somatostatin was applied via a macropipette lowered into the recording bath near the surface of the cell under study. The macropipette was withdrawn from the bath to terminate somatostatin application.

#### b. Calcium-activated chloride current:

A Ca<sup>++</sup>-dependent Cl<sup>-</sup> current has been observed in several mammalian and non-mammalian cell types. To understand the role of this current in the regulation of cell excitability in secretory cells, we have investigated the mechanisms that underly its activation and deactivation in AtT-20 mouse pituitary cells. The Ca<sup>++</sup>-dependent Cl<sup>-</sup> current can be activated in AtT-20 cells by step-depolarization into the range that activates the voltage-activated Ca<sup>++</sup> current. In the studies described below, we have used the whole-cell patch clamp technique to examine the roles of voltage and Ca<sup>++</sup> in the activation of the Cl<sup>-</sup> current. The patch-clamp methods used were similar to those described above except for the external and internal solutions. The main solutions used in this study are listed in the following table.

#### External Solutions (mM).

	NaCl	TEA-Cl	CaCl <sub>2</sub>	MgCl <sub>2</sub>	HEPES	glucose
1.	150		5	2.0	10	20
2.	146		2	10.0	10	20
3.	155		2	0.8	10	20
4.		156	5	2.0	10	20
5.		160	5	0.8	10	20
6.		158	2	2.0	10	20

#### Internal Solutions (mM)

	CsCl	Cs <sub>2</sub> SO <sub>4</sub>	HEPES	EGTA	BAPTA	CaCl <sub>2</sub>	ATP Regenerating System*
1.	165		10	0.2			
2.	165		10				
3.	165		10		5	2.38	
4.	50	90	10				
5.	30	117	10				
6.	150		10				+
7.	130		10		5	0.42	+

Solutions were adjusted to pH 7.36 with NaOH (external solutions 1-3) or CsOH (external solutions 4-6, internal solutions 107). Osmolality ranged from 335-340 mOsm/kg (external) and 309-320 mOsm/kg (internal).

\*The ATP regenerating system consisted of 4 mM ATP-Mg, 20 mM creatine phosphate and 50 units/ml creatine kinase.

## 2. PC12 cells:

The rat chromaffin cell line, PC12, has many characteristics in common with sympathetic neurons. In addition to nicotinic receptors, whose activation stimulates secretion, muscarinic binding sites have been identified on PC12 cells (Nature 297: 152, 1982). However, little is known regarding the functional significance of these muscarinic binding sites. We studied the effect of muscarinic agonists on intracellular  $\text{Ca}^{2+}$  mobilization, phosphoinositide metabolism and transmitter release from PC12 cells. Intracellular free  $\text{Ca}^{2+}$  was measured using the fluorescent  $\text{Ca}^{2+}$  indicator Quin2. Cells were loaded with 10  $\mu\text{M}$  Quin2 AM for 20 min, washed and resuspended at concentration of  $2 \times 10^6$  cells/ml in HEPES buffered saline containing 1.8 mM  $\text{Ca}^{2+}$ . Phosphoinositide metabolism was studied by prelabeling cells with [ $^3\text{H}$ ] inositol (5  $\mu\text{Ci}/\text{ml}$ ) for 24 hr. To investigate neurotransmitter release, cells were preloaded for 30 min. with [ $^3\text{H}$ ] norepinephrine (500 nM, 23.1 Ci/mmol).

## 3. Pineal cells:

Cells in the pineal gland secrete the hormone melatonin and it has been reported that ethanol administration affects the secretion of melatonin. To determine the membrane events associated with secretion, we first characterized the membrane currents in pineal cells. Membrane currents of dispersed pineal cells were studied using the whole-cell patch clamp recording technique. Single cells from the pineal gland were acutely separated with enzymatic techniques from male rats (200-300 g) kept under LD 12:12 with lights on from 6 A.M. to 6 P.M. The artificial cerebrospinal fluid contained (mM): 150 NaCl; 5.4 KCl; 2  $\text{CaCl}_2$ ; 1  $\text{MgCl}_2$ ; and 10 HEPES; pH 7.4 and internal solution was composed of (mM): 130 KCl; 1  $\text{CaCl}_2$ ; 2  $\text{MgCl}_2$ ; 10 HEPES; and 11 EGTA.

### Major Findings:

#### 1. AtT-20 cells:

##### a. Calcium current:

AtT-20 cells were voltage-clamped at a holding potential of -80 mV and stepped from -120 to +80 mV using steps 100 ms in duration. Cell input resistance, measured between -120 and -60 mV, was 2.0-7.9 Gohm. An inward current activated rapidly at potentials positive to -40 mV, peaked in 6-7 ms, inactivated slowly, was inhibited by 2mM  $\text{Co}^{2+}$ , and was present in 10 mM  $\text{Ba}^{2+}$ . I-V curves for this voltage-dependent  $\text{Ca}^{2+}$  current ( $I_{\text{Ca}}$ ) were obtained by measuring  $I_{\text{Ca}}$  at peak amplitude. Somatostatin ( $10^{-8}$  to  $10^{-6}\text{M}$ ) was applied from a macropipette lowered into the external solution near the cell under study. Somatostatin decreased peak  $I_{\text{Ca}}$  by  $30.1 \pm 6.7\%$  ( $n=6$ ) during voltage steps eliciting maximum current amplitude (+10 to +15mV). Within two min after beginning somatostatin washout,  $I_{\text{Ca}}$  returned to control level. Control solutions of peptide vehicle (acidified bovine serum) diluted with external solution (identical to the peptide dilutions) had no apparent effect on  $I_{\text{Ca}}$ . The results suggest that the somatostatin-induced inhibition of ACTH secretion may result from the reduction of calcium current by somatostatin.

We also tested whether guanine nucleotide-binding proteins (N or G proteins) are involved in the signal transduction mechanism between the somatostatin receptor and the voltage-gated  $\text{Ca}^{2+}$  channel. Treatment of the cells with pertussis toxin, which blocks the function of the GTP-binding proteins  $\text{N}_i$  and  $\text{N}_o$  by ADP-ribosylation, completely abolished the action of somatostatin on both  $I_{\text{Ca}}$  and intracellular



$\text{Ca}^{2+}$ . Intracellular application of the nonhydrolyzable guanine nucleotide analog guanosine 5'-(3-O-thio)triphosphate (GTP $\gamma$ -S), which irreversibly activates N proteins, changed the somatostatin effect on  $\text{I}_{\text{Ca}}$  from a reversible to an irreversible inhibition. Intracellular GTP- $\gamma$ -S alone caused a very slowly developing inhibition of  $\text{I}_{\text{Ca}}$ . When  $\text{I}_{\text{Ca}}$  was inhibited by GTP- $\gamma$ -S (alone or with somatostatin), it failed to respond to subsequent applications of somatostatin. The effect of GTP- $\gamma$ -S on the inhibition of  $\text{I}_{\text{Ca}}$  by somatostatin was not altered by the intracellular application of cAMP and IBMX. The results indicate that a GTP-binding protein is directly involved in the cAMP-independent, receptor mediated inhibition of voltage-dependent  $\text{Ca}^{2+}$  channels.

The role of protein kinase C in regulating  $\text{Ca}^{2+}$  channel activity was also investigated using the whole-cell patch-clamp technique in AtT-20 cells. Extracellular application of the protein kinase C activator 1-oleoyl-2-acetyl-glycerol (OAG) reduced voltage-dependent  $\text{Ca}^{2+}$  current. This effect was reversible and dose-dependent (10-100  $\mu\text{M}$ ). Pertussis toxin did not block the effect of OAG on  $\text{Ca}^{2+}$  current suggesting that OAG does not affect  $\text{Ca}^{2+}$  channels via a pertussis toxin sensitive GTP-binding protein.  $\text{Na}^+$  free solutions did not block the effect of OAG on  $\text{Ca}^{2+}$  channels suggesting that this effect of OAG does not involve the  $\text{Na}^+/\text{H}^+$  antiporter. The phorbol esters 12-deoxyphorbol 13-isobutyrate (10  $\mu\text{M}$ ) and phorbol 12,13-diacetate (100  $\mu\text{M}$ ) also reduced  $\text{Ca}^{2+}$  current. The results suggest that protein kinase C may be an inhibitory regulator of voltage-dependent  $\text{Ca}^{2+}$  channels.

#### b. Calcium-activated chloride current:

Voltage-clamp recordings of the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current were also made from AtT-20 cells using the whole-cell patch-clamp technique. Cells were perfused internally with  $\text{Cs}^+$  to block  $\text{K}^+$  currents and bathed externally with either 1  $\mu\text{M}$  tetrodotoxin or with tetraethylammonium (TEA) as a  $\text{Na}^+$ -substitute to block voltage-activated  $\text{Na}^+$  currents. Depolarizing voltage steps from a holding potential of -80 mV to potentials positive to -30 mV evoked two currents: a fast inward current that activated between -30 mV and +70 mV and a slowly-activating current (designated "slow step current") that was inward between -30 mV and near 0 mV (the  $\text{Cl}^-$  equilibrium potential), and outward positive to about 0 mV. Repolarization to -80 mV revealed a slowly decaying, inward tail current, whose magnitude with respect to step potential closely matched the current-voltage (I-V) relationship of the voltage-activated  $\text{Ca}^{++}$  current. Activation of the fast inward current, slow step current, and tail current, was prevented by extracellular application of  $\text{Cd}^{++}$  or removal of extracellular  $\text{Ca}^{++}$ . Replacement of extracellular  $\text{Ca}^{++}$  with  $\text{Ba}^{++}$  potentiated the fast inward current but blocked the slow step and tail currents. Intracellular perfusion with greater than 1 mM of the  $\text{Ca}^{++}$  chelators EGTA or BAPTA prevented activation of the slow step and tail currents, but not the fast inward current. The reversal potential of the slow inward current was sensitive to changes in the  $\text{Cl}^-$  equilibrium potential but not to substitution of TEA for  $\text{Na}^+$ . The slow step current but not the fast inward current was partially blocked by the  $\text{Cl}^-$  channel blocker, SITS. These data indicate that both the slow inward tail current and the slowly-activating, reversible step current were a  $\text{Ca}^{++}$ -dependent  $\text{Cl}^-$  current. The fast inward current was a voltage-activated  $\text{Ca}^{++}$  current. In the absence of intracellular EGTA, the tail current decayed with complex kinetics, its timecourse apparently dependent on the magnitude of the voltage-activated  $\text{Ca}^{++}$  current. In the presence of 200  $\mu\text{M}$  intracellular EGTA, the tail current decayed significantly faster and often decayed exponentially. Intracellular EGTA greatly reduced the apparent dependence of tail current duration on the magnitude of the voltage-activated  $\text{Ca}^{++}$  current. In the absence of

intracellular EGTA, replacement of extracellular  $\text{Na}^+$  with TEA or tetramethylammonium (TMA) increased the amplitude of and prolonged the tail current. Intracellular perfusion with 200  $\mu\text{M}$  EGTA prevented the prolongation but not the amplitude increase that followed  $\text{Na}^+$  substitution. The timecourse of tail current decay was mildly voltage-dependent. Thus voltage-dependence remained following intracellular perfusion with 200  $\mu\text{M}$  EGTA or replacement of extracellular  $\text{Na}^+$  with TEA. The  $\text{Ca}^{++}$ -dependent  $\text{Cl}^-$  current was not activated when intracellular  $\text{Ca}^{++}$  was buffered at 0.1  $\mu\text{M}$  or less. With internal  $\text{Ca}^{++}$  buffered between 0.5 and 1.0  $\mu\text{M}$  and  $\text{Ca}^{++}$  omitted from the external solution, the  $\text{Cl}^-$  current appeared to be activated at membrane potentials between -80 and -50 mV. Under these conditions of constant  $\text{Ca}^{++}$  concentrations, membrane depolarization resulted in additional  $\text{Cl}^-$  current activation. These data suggest that the complex decay kinetics of the  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  current are largely due to mechanisms that modulate intracellular  $\text{Ca}^{++}$  levels. At least one of these mechanisms is dependent on extracellular  $\text{Na}^+$ . The decay timecourse is also mildly voltage-sensitive, and this voltage-sensitivity appears to be independent of mechanisms that modulate intracellular  $\text{Ca}^{++}$  levels. Finally, buffering intracellular  $\text{Ca}^{++}$  at an elevated level is sufficient to activate the slow  $\text{Cl}^-$  current. Moreover, in the presence of 0.5 to 1.0  $\mu\text{M}$  intracellular  $\text{Ca}^{++}$ , activation of the  $\text{Ca}^{++}$ -dependent  $\text{Cl}^-$  current is voltage-sensitive.

## 2. PC12 cells:

When PC12 cells were exposed to muscarine, the cells rapidly responded with elevation of cellular inositol trisphosphate levels, elevation of intracellular  $\text{Ca}^{2+}$  and release of stored transmitter. These three phenomena were totally inhibited by the muscarinic antagonist, atropine, but were unaffected by the nicotinic antagonist, d-tubocurarine. Muscarine-stimulated increases in inositol trisphosphate, intracellular  $\text{Ca}^{2+}$  and neurotransmitter release displayed similar time courses and concentration-dependencies suggesting that the secretion observed may be associated with the formation of inositol trisphosphate and elevation of intracellular  $\text{Ca}^{2+}$ . The increase in intracellular  $\text{Ca}^{2+}$  appeared to be due to a mobilization of  $\text{Ca}^{2+}$  from intracellular stores since the increase in intracellular  $\text{Ca}^{2+}$  was not inhibited by the voltage-dependent  $\text{Ca}^{2+}$  antagonist, nifedipine, at concentrations demonstrated to block  $\text{K}^+$ -induced  $\text{Ca}^{2+}$  influx into the cells, and no uptake of  $^{45}\text{Ca}^{2+}$  was noted when cells were stimulated with muscarine. Elevation of inositol trisphosphate, intracellular  $\text{Ca}^{2+}$  and stimulation of transmitter release were, however, partially dependent on the presence of extracellular  $\text{Ca}^{2+}$ . The results suggest that muscarine-stimulated release of neurotransmitter may be associated with an inositol trisphosphate-induced mobilization of intracellular  $\text{Ca}^{2+}$ .

The effect of ethanol on muscarine-stimulated release of  $[\text{H}^3]$ -1-norepinephrine ( $[\text{H}^3]$ NE) was also studied in PC12 cells. At concentrations of 25 mM and above, ethanol produced a dose-dependent inhibition of muscarine-stimulated release of  $[\text{H}^3]$ NE. The inhibition of muscarine-stimulated transmitter release occurred in the absence of any apparent effect of ethanol on  $[\text{H}^3]$ NE uptake or on muscarinic binding to the cells. Muscarinic stimulation also elevated intracellular free  $\text{Ca}^{2+}$  and this elevation was inhibited by ethanol. At concentrations greater than 100 mM, ethanol produced an increase in the basal release of  $[\text{H}^3]$ NE. Intracellular free  $\text{Ca}^{2+}$  was also increased by ethanol concentrations greater than 100 mM. The results suggest that the effects of ethanol on neurotransmitter release are associated with ethanol effects on intracellular free  $\text{Ca}^{2+}$ .

### 3. Pineal Cells:

Membrane currents of dispersed pineal cells were studied using the whole-cell patch clamp recording technique. The macroscopic ionic current observed in artificial cerebrospinal fluid was dominated by an outward current component with little or no apparent inward current. Study of the outward component in solution without added  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  revealed the existence of two distinct outward currents. Depolarizations from a holding potential of -100 mV activated a fast current which reached a peak within 15 ms and completely decayed in about 150 ms. This current activated at potentials more positive than -50 mV and displayed steady state inactivation at depolarizing voltages with half-inactivation near -80 mV. The second outward current isolated from a holding potential of -50 mV activated at potentials positive to -20 mV, reached a steady state current amplitude within 50 ms and was sustained up to 400 ms. In the presence of 2 mM external  $\text{Ca}^{2+}$ , the I-V relationship did not display a region of negative slope conductance (N-shape) suggesting that  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current did not contribute significantly to the outward current. In solutions designed to isolate calcium currents a small inward current (<100 pA) was observed. It had a threshold at -40 mV and its amplitude reached a peak at about -10 mV. We conclude that acutely dissociated pineal cells display two distinct K currents: (i) a transient current similar to the A current ( $I_A$ ); and (ii) a slowly activating, sustained current similar to the delayed rectifier current ( $I_K$ ).

The effect of ethanol on these neurosecretory mechanisms is currently being investigated.

#### Significance to Biomedical Research and the Program of the Institute:

The cellular mechanisms involved in ethanol's actions in nervous and neuroendocrine tissues are poorly understood. Characterization of synaptic and neurosecretory mechanisms and the effects of ethanol on those mechanisms holds the promise of increasing our understanding of the cellular basis of ethanol's actions in these tissues.

#### Proposed Course:

Synaptic and neurosecretory mechanisms will be characterized more fully, and the actions of ethanol on those mechanisms will be investigated more extensively. In addition, the actions of other alcohols and CNS depressants such as barbiturates, opiates and benzodiazepines will be characterized and compared to ethanol.

#### Publications:

Cordingley, G.E. and Weight, F.F.: Non-cholinergic synaptic excitation in neostriatum: pharmacologic evidence for mediation by a glutamate-like transmitter. British J. Pharmacol., 88:847-856, 1986.

Lewis, D.L., Weight, F.F. and Luini, A.: A guanine nucleotide-binding protein mediates the inhibitory action of somatostatin on voltage-dependent calcium channels in pituitary cell line. Proc. Nat'l. Acad. Sci., 83:9035-9039, 1986.

- Luini, A. Lewis, D.L., Guild, S., Schofield, G.G. and Weight, F.F.: Somatostatin an inhibitor of ACTH secretion, decreases cytosolic free calcium and voltage dependent calcium current in a pituitary cell line. J. Neuroscience., 6:3128-3132, 1986.
- Smith, P.A. and Weight, F.F.: The pathway for the slow inhibitory post-synaptic potential in bullfrog sympathetic ganglia. J. Neurophysiol., 56:823-834, 1986.
- Yavari, P. and Weight, F.F.: Antagonists discriminate muscarinic excitation and inhibition in sympathetic ganglion. Brain Research, 400:133-138, 1987.
- Korn, S.J. and Weight, F.F.: Patch-clamp study of the calcium-dependent chloride current in AtT-20 pituitary cells. J. Neurophysiol., in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00480-04 LPPS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nerve Cell Excitability and Ethanol Actions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: F. F. Weight Section Chief LPPS, NIAAA

Others: J. E. Freedman Staff Fellow LPPS, NIAAA  
 S. R. Ikeda Senior Staff Fellow LPPS, NIAAA  
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## COOPERATING UNITS (if any)

Howard Hughes Medical Inst., Columbia Univ. (A. B. MacDermott); Dept. of Physiology, Armed Forces Radiobiol. Research Inst. (K. L. Zbicz)

## LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

## SECTION

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NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

3.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Although it is well known that the administration of ethanol can affect nervous system excitability, the cellular basis of such actions is poorly understood. The objective of this project was to characterize the mechanisms regulating nerve cell excitability and the effects of ethanol on those mechanisms. The membrane mechanisms that underlie excitable phenomena were characterized by electrophysiological methods. The membrane ion currents that are involved in the regulation of action potential generation and the steady-state excitability of the membrane have been investigated in neurons from the superior cervical and nodose ganglia and in the hippocampal region of brain. In the neurons from nodose ganglion, two different sodium currents have been characterized, a tetrodotoxin (TTX)-sensitive sodium current and a TTX-resistant sodium current. The TTX-resistant sodium current has a monovalent cation selectivity that is similar to that of the TTX-sensitive sodium current. The TTX-resistant sodium current is less sensitive to divalent cations than the calcium current. These neurons also have two calcium currents, transient and sustained. Investigation of potassium currents in the sympathetic neurons has revealed at least 3 different currents: a voltage-activated transient current, a delayed rectifier current and a sustained calcium-activated current. Similar potassium currents have been characterized in CA3 pyramidal neurons from hippocampus. Studies of transmitter regulation of these currents has revealed that the sustained calcium-activated potassium current in hippocampal neurons is inhibited by muscarinic receptor activation, and the calcium current in sympathetic neurons is inhibited by somatostatin. The effect of ethanol is currently being studied on these currents. The significance of the project lies in the fact that the identification of the mechanisms involved in nerve cell excitability and the investigation of the action of ethanol on those mechanisms holds the promise of increasing our understanding of the cellular basis of ethanol's actions in the nervous system.

PROJECT DESCRIPTION:Investigators:

P. I.:	F. F. Weight	Section Chief	LPPS, NIAAA
Others:	J. E. Freedman	Staff Fellow	LPPS, NIAAA
	S. R. Ikeda	Senior Staff Fellow	LPPS, NIAAA
	A. B. MacDermott	Research Scientist	H.H.M.I., Columb. U.
	G. G. Schofield	Senior Staff Fellow	LPPS, NIAAA
	G. G. White	Staff Fellow	LPPS, NIAAA
	K. L. Zbicz	Research Scientist	DP, AFRI

Objectives:

Although it is well known that the administration of ethanol can affect nervous system excitability, the cellular basis of such actions is poorly understood. The objectives of this project were to characterize the mechanisms regulating nerve cell excitability and the effects of ethanol on those mechanisms.

Methods Employed:

Excitability mechanisms were characterized by two electrophysiological methods: (1) whole cell patch clamp recording of acutely isolated adult rat nodose or superior cervical ganglion cells; and (2) single-electrode voltage clamp recording of CA3 pyramidal neurons in hippocampal slice.

## 1. Patch clamp experiments:

Cell isolation procedure: Single nodose or superior cervical ganglion somata were acutely isolated by enzymatic dispersion from male and female Sprague Dawley rats (100 - 300 g). The rats were decapitated with a laboratory guillotine and the heads placed in iced Hank's balanced salt solution (HBSS). The ganglia were removed and placed modified HBSS supplemented with 6.5 g/l glucose and 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.4. Each ganglion was cleaned of connective tissue and minced with fine iridectomy scissors. The tissue fragments were then transferred to 5 ml of modified HBSS containing 1 mg/ml trypsin (type III), 1 mg/ml collagenase (type IA), and 0.1 mg/ml DNAase (type III). The tissue fragments were incubated for 1 hr at 35°C in a 25 cm<sup>2</sup> tissue culture flask which allowed microscopic inspection of the tissue fragments. After incubation, the flask was shaken vigorously which released the cell somata from the ganglion fragments. The enzyme solution containing the dispersed cells was then inhibited by the addition of 7 ml of modified HBSS containing 2 mg/ml soya bean trypsin inhibitor (type II-S), 1 mg/ml bovine serum albumin (BSA), 10% fetal calf serum (Gibco), and 5 mM CaCl<sub>2</sub>. The cell suspension was then added to 35 mm poly-L-lysine coated tissue culture dishes and superfused with physiological saline. The enzymes and inhibitor were obtained from Sigma Chemical Co. St. Louis Mo. and prepared daily.

Patch clamp recording: Microelectrodes were fabricated from borosilicate glass

capillaries (1.69 mm od, 1.35 mm id; Drummond Scientific Co., Broomall, Pa.) using a Narashige PW-6M microelectrode puller, coated with Sylgard® (Dow Corning Corp., Midland, MI) to reduce the electrode capacitance and the tip fire polished on a microforge. Pipettes filled with internal solutions had resistances in the range 0.3-2 Mohms. The cells were voltage clamped using a L/M-EPC7 patch clamp amplifier (List Electronic, West Germany). Patch electrodes were sealed against the membrane by suction yielding seal resistances greater than 10 Gohms. A holding potential of -50 mV was applied to the pipette and the electrode capacitance neutralized. The membrane patch was then disrupted by further suction after which the membrane capacitance and series resistance controls were optimally adjusted. The cells were then clamped at the selected holding potential and a series of hyperpolarizing and depolarizing command potentials were delivered. Membrane currents were filtered using a 4 pole Bessel filter (3KHz - 3dB) (Krohn-Hite 3750), digitized with a 12 bit A/D converter, and stored for analysis using a PDP-11/23 microcomputer. Records were digitized at 100 usec/point ( $\text{Na}^+$  currents) or 150 usec/point ( $\text{Ca}^{++}$  currents) unless otherwise stated. Current traces and current/voltage relationships were corrected for linear leakage current measured from hyperpolarizing command pulses. Test solutions were applied by lowering a large bore (>10 um) pipette, containing the test solution, close to the cell soma. All experiments were conducted at 19 to 23°C.

## 2. Single-electrode voltage clamp experiments:

**Brain slice preparation:** Slices were prepared from the hippocampus, from male Hartley guinea pigs (300-600 g). The hippocampus was cooled by immersion in iced artificial cerebrospinal fluid (CSF) and slices (400-450 um) were prepared on a drop-blade tissue chopper (Sorval). The slices were immediately placed in a prechamber for later use. The prechamber was designed to maintain slices at room temperature in constantly circulating artificial CSF that had been equilibrated with 95%  $\text{O}_2$ -5%  $\text{CO}_2$ . Slices were kept in the prechamber for a minimum of 1 h, allowing the tissue to stabilize before recording was performed. A single slice was then positioned in a recording chamber and held submerged in the artificial CSF equilibrated with a gas mixture of 95%  $\text{O}_2$ -5%  $\text{CO}_2$ .

**Solutions:** The artificial CSF used had the following composition (mM):  $\text{NaCl}$  124,  $\text{KCl}$  3.2,  $\text{CaCl}_2$  2.4,  $\text{MgCl}_2$  1.3,  $\text{NaHCO}_3$  26,  $\text{NaH}_2\text{PO}_4$  1.2, glucose 10. The pH was 7.4 after bubbling with 95%  $\text{O}_2$ -5%  $\text{CO}_2$ . Tetrodotoxin (TTX,  $2 \times 10^{-7}$  M) was present in all experiments to prevent sodium spikes from occurring during depolarizing steps. In experiments in which  $\text{MnCl}_2$  or  $\text{CdCl}_2$  was added to the solution,  $\text{NaH}_2\text{PO}_4$  and  $\text{MgSO}_4$  were omitted. When studying the effects of  $\text{Mn}^{2+}$  or  $\text{Cd}^{2+}$ ,  $\text{MgCl}_2$  was added to the control solution to keep the divalent ion concentration constant throughout the experiment.

**Voltage-clamp:** A single-microelectrode voltage current clamp (SEC) based on the design of Wilson and Goldner was used for intracellular potential measurements and voltage clamping of the neuronal membrane. The switching frequency of the clamp was set at 4-5 kHz with current passed during 25% of the cycle. We used relatively low resistance microelectrodes fabricated on a Brown-Flaming microelectrode puller using thin-walled borosilicate tubing (ID 0.9, OD 1.2 mm;

Fredrick Haer). Such microelectrodes typically had resistances of 15-30 Mohms when filled with 3 M CsCl and had tip lengths (i.e., the distance from the initial narrowing of the glass to the tip) of 8-12 mm. In comparison with higher-resistance microelectrodes, these microelectrodes allowed more current to be passed and allowed the clamp to be operated at a higher gain, reducing discrepancies between the command potential and actual potential of the cell. Data acquisition: The SEC output was connected to an LSI-11/23 microcomputer system, and data was digitized and stored on magnetic media for subsequent analysis. The current signal was first passed through a low-pass Butterworth filter (24 dB/decade) with the corner frequency set at 300 Hz for 1-s voltage steps and at 750 Hz when observing faster events during 100-ms steps. The microcomputer was also used to generate the voltage commands.

### Major Findings:

1. Whole cell patch-clamp recording of nodose and sympathetic ganglion cells: The electrical properties of nodose ganglion cells acutely isolated from adult rats were studied using the whole cell patch-clamp recording method. Current clamp recordings revealed a mean resting membrane potential of -54.3 mV and an input resistance of 527 Mohms. Depolarizing current steps evoked action potentials with the following properties (mean): amplitude 111 mV, threshold -36mV, and rate of rise 117 V/sec. Two types of action potentials were observed, short duration and long duration. These properties, with the exception of input resistance (527 Mohms cf. 50 Mohms), are similar to those reported previously using intracellular recording methods in intact nodose ganglia. Brief application of 10  $\mu$ M 5-hydroxytryptamine (5-HT) resulted in a rapid depolarization and burst of action potentials in the majority of cells. With voltage-clamp recording, step depolarizations to potentials positive to -15 mV elicited a transient inward current which was followed by a sustained outward current. Inward  $\text{Na}^+$  current was isolated by ion substitution and pharmacological agents. Two types of  $\text{Na}^+$  current were observed. One current was completely abolished by 3 to 15  $\mu$ M tetrodotoxin (TTX), had a rapid time course, activated over the potential range -70 to -10 mV, and attained half-maximal conductance at -30 mV. The other current persisted in the presence of 15  $\mu$ M TTX, had a slower time course, activated over the potential range -30 to 0 mV, and attained half-maximal conductance at -10 mV. In addition, 500  $\mu$ M  $\text{Cd}^{++}$  and 5.0 mM  $\text{Co}^{++}$  reduced the TTX-insensitive current to 53 and 42% of control respectively. Inward  $\text{Ca}^{++}$  current was isolated by ion substitution and pharmacological agents, and identified by a dependence on external  $\text{Ca}^{++}$ .  $\text{Cd}^{++}$  (500  $\mu$ M) and  $\text{Co}^{++}$  (5 mM) reduced the maximal inward current to 5% and 20% of control respectively. When  $\text{Ba}^{++}$  was substituted for  $\text{Ca}^{++}$  as the charge carrier, the maximal inward current increased to 175% of control. Some cells had two  $\text{Ca}^{++}$  current components, an inactivating component which activated near -60 mV and a large sustained current which activated near -40 mV. The initial inactivating current appeared as a "hump" on the I/V curve over the potential range -60 to -30 mV. The results indicate that some cells have more than one type of  $\text{Na}^+$  and/or  $\text{Ca}^{++}$  channels.

Monovalent cation selectivity and divalent cation sensitivity of the tetrodotoxin (TTX)-resistant  $\text{Na}^+$  current in dissociated adult rat nodose ganglion neurones were investigated using the whole-cell patch-clamp technique. The TTX-resistant  $\text{Na}^+$  current was isolated using ion substitution and pharmacological agents. Under these conditions, the current reversal potential shifted 52 mV per tenfold change in external  $[\text{Na}^+]$ . Inorganic and organic monovalent cation permeability ratios ( $P_X/P_{\text{Na}}$ ) were determined from changes in reversal potential and the Goldman-



Hodgkin-Katz equation. The  $P_{X/P_{Na}}$  values determined by the former method were  $HONH_3^+$ , 1.38;  $Li^+$ , 1.00;  $H_2NNH_3^+$ , 0.66;  $NH_4^+$ , 0.28;  $CH_3NH_3^+$ , <0.13;  $K^+$ , <0.13;  $R^+$ , >0.12;  $Cs^+$ , <0.10;  $(CH_3)_4N^+$ , <0.10. The values determined by either method agreed within 10%. The effects of  $Cd^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$  and  $Ni^{2+}$  on the TTX-resistant  $Na^+$  current were analysed from peak-conductance values. These ions shifted the activation of the current to more positive potentials and decreased the maximal conductance. At 3 mM concentrations  $Cd^{2+}$ ,  $Ni^{2+}$ ,  $Co^{2+}$ , and  $Mn^{2+}$  decreased the maximal conductance 64.6, 50.7, 25.0 and 20.3% respectively. The results indicate that: (a) the monovalent cation selectivity of the TTX-resistant  $Na^+$  current is similar to that of the TTX-sensitive  $Na^+$  current in other tissues; and (b) the TTX-resistant  $Na^+$  current is less sensitive to divalent cations than the  $Ca^{2+}$  current in these neurones. These observations suggest that the structure determining the monovalent cation permeability of the TTX-resistant  $Na^+$  current is similar to that of the TTX-sensitive  $Na^+$  current in other tissues, and that the channels carrying the TTX-resistant  $Na^+$  current are distinct from those responsible for the  $Ca^{2+}$  current.

Potassium currents were investigated in superior cervical ganglion cells in solutions designed to isolate  $K^+$  current. Depolarizations from a holding potential of -80 mV elicited two distinct outward current components. An initial component activated at potentials more positive than -60 mV, rose rapidly, and then decayed. A second sustained current component was observed at potentials more positive than -20 mV. The initial transient current was inactivated by holding the membrane potential at -50 mV, whereas the second current component was reduced only slightly at this holding potential. The transient current decayed with a time constant of approximately 20 msec. When the holding potential was -50 mV, the transient current could be fully reactivated by a conditioning prepulse to -100 mV. The time constant of reactivation at -100 mV was approximately 36 msec. This current was not blocked by 0.5 mM  $Cd^{2+}$ -containing or  $Ca^{2+}$ -free external solutions, but could be markedly reduced by 2 mM 4-aminopyridine. The transient current had the characteristics of  $I_A$ . The sustained outward current could be further separated into two outward current components. Depolarizations from -50 mV elicited a current that activated at potentials more positive than -20 mV, and was blocked by superfusion with a 0.5 mM  $Cd^{2+}$ -containing or  $Ca^{2+}$ -free external solution. Digital subtraction revealed that the  $Ca^{2+}$ -sensitive current activated with an exponential time constant of 20 to 40 msec over the potential range 0 to +10 mV. The current that remained in the  $Ca^{2+}$ -free external solution also activated at potentials more positive than -20 mV and was reduced by superfusion with 10 mM TEA. Under the conditions of these experiments there appear to be at least 3 outward currents: (i) a transient  $K^+$  current with characteristics similar to  $I_A$ ; (ii) a sustained  $Ca^{2+}$ -sensitive  $K^+$  current similar to  $I_{K(Ca)}$ ; and (iii) a sustained  $Ca$ -insensitive, TEA-sensitive  $K^+$  current that appears to be similar to the delayed rectifier or  $I_K$ .

Somatostatin-like immunoreactivity has been reported to occur in the postganglionic neurons of sympathetic ganglia. We therefore have investigated the effect of somatostatin (SOM) on the  $Ca^{2+}$  current in sympathetic neurons. Voltage-clamp recordings, using the whole-cell patch-clamp technique, were made from acutely isolated adult rat superior cervical ganglion (SCG) neurons in solutions (external and internal) designed to isolate  $Ca^{2+}$  current evoked from a holding potential of -80 mV. The concentration-response relationship for SOM could be fitted to a single-site binding model with an apparent dissociation constant of 121 nM; the maximal attainable block of  $Ca^{2+}$  current by SOM was 50%. SOM also produced a pronounced slowing of the  $Ca^{2+}$  current rising phase, especially at more

depolarized potentials. At higher concentrations (0.03 - 1.0 M), prolonged application of SOM resulted in a progressive decrease in blocking ability. The results are consistent with a neurotransmitter and/or neuromodulator role for SOM in the sympathetic nervous system.

2. Single-electrode voltage-clamp recording of CA3 pyramidal neurons: Inward currents in hippocampal CA3 pyramidal neurons were studied using the single microelectrode voltage-clamp (SEC) technique. Neurons in the pyramidal layer of region CA3 were impaled with a single microelectrode containing 3M CsCl. Diffusion of  $\text{Cs}^+$  into the neurons produced a large reduction in outward  $\text{K}^+$  currents, revealing a depolarization activated inward current. This current could be reduced by the application of  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ , or  $\text{Ca}^{2+}$ -free solutions, and is therefore presumed to be mediated by  $\text{Ca}^{2+}$ . In  $\text{Cs}^+$  loaded neurons the inward current activated rapidly (<30 msec) and gradually decayed with maintained depolarization. This decline in inward current was incomplete, however, and the recorded current often remained net inward during a depolarization lasting several seconds. The data indicate that the inward  $\text{Ca}^{2+}$  current rapidly activates and that a fraction of this current inactivates with maintained depolarization. This inactivation may not be dependent on  $\text{Ca}^{2+}$  since it occurred when  $\text{Ba}^{2+}$  was substituted for  $\text{Ca}^{2+}$  in the artificial CSF used. The deactivation time course for the inward current suggests that two or more types of  $\text{Ca}^{2+}$  channels are present in these cells.

Outward currents were also studied in Cs-loaded hippocampal CA3 pyramidal neurons. Several outward currents that have been previously observed in these neurons were significantly reduced or blocked by  $\text{Cs}^+$  loading. These include a transient  $\text{Ca}^{2+}$ -activated current, a transient voltage-activated current, and a current similar to the delayed rectifier. Step depolarizations to potentials positive to -14 mV elicited a slowly developing outward current. The slowly developing outward current was greatly reduced or blocked by the application of 3 mM  $\text{Co}^{2+}$ , 2-4 mM  $\text{Mn}^{2+}$ , or  $\text{Ca}^{2+}$ -free external solution. The slowly developing outward current was also reduced by the application of 10-25 mM tetraethylammonium (TEA) or 0.2-1 mM  $\text{Ba}^{2+}$ . Application of 500  $\mu\text{M}$  4-aminopyridine (4-AP) had no apparent effect on this current. As the holding potential was made more positive over a range of -60 to -20 mV, the amplitude of this outward current decreased. Application of muscarine or carbachol reversibly depressed the slow outward current. However, in  $\text{Ca}^{2+}$ -free solution containing  $\text{Mn}^{2+}$  or  $\text{Co}^{2+}$ , an effect of muscarine or carbachol on outward current was not observed. The results suggest that the slowly developing outward current is a  $\text{Ca}^{2+}$ -dependent current that is inhibited by activation of muscarinic receptors. The reduction of outward current by muscarinic receptor activation may result from the reduction of  $\text{Ca}^{2+}$  current that was also observed in response to muscarinic agonists.

The effect of ethanol on the above ion currents is currently being studied.

#### Significance to Biomedical Research and the Program of the Institute:

The behavioral effects of ethanol and the development of dependence and tolerance to ethanol are due to the actions of ethanol on the nervous system. The cellular basis of such actions, however, is poorly understood. Characterization of the cellular mechanisms that regulate nerve cell excitability and the actions of ethanol on those mechanisms holds the promise of increasing our understanding of the cellular basis of ethanol's actions in the nervous system.

Proposed Course:

The mechanisms regulating nerve cell excitability will be characterized more fully, and the actions of ethanol on those mechanisms will be investigated more extensively. In addition, the actions of other alcohols and CNS depressants such as barbiturates, opiates, and benzodiazapines will be characterized and compared to ethanol.

Publications:

Ikeda, S.R. and Schofield, G.G.: Tetrodotoxin-resistant sodium current of rat nodose neurones: monovalent cation selectivity and divalent cation sensitivity. J. Physiol. (Lond), in press.

Ikeda, S.R., Schofield, G.G. and Weight, F.F.: Somatostatin blocks a calcium current in acutely isolated adult rat superior cervical ganglion neurons. Neuroscience Letters, in press.

Zbicz, K.L. and Weight, F.F.: Postsynaptic mechanisms in the potentiation of synaptic responses. Experimental Brain Research, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00472-05 LPPS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ethanol Effects on the Immune System

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Marietta                      Physiologist                      LPPS, NIAAA

Others: F. Weight                      Section Chief                      LPPS, NIAAA  
 M. Eckardt                      Section Chief                      LCS, NIAAA  
 A. Parfitt                      Biochemist                      LPPS, NIAAA

## COOPERATING UNITS (if any)

Department of Rickettsial Diseases, Walter Reed Army Institute of Research  
 (T.R. Jerrells, D. Peritt, W. Smith)

## LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

## SECTION

Section on Immunology

## INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

## TOTAL MAN-YEARS:

0.7

## PROFESSIONAL:

0.1

## OTHER:

0.6

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects                      ☐ (b) Human tissues                      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Alcoholics are known to be very susceptible to infections. We studied the effects of alcohol on the immune system of rats and mice using various models to produce physical dependence. Both normal and adrenalectomized animals were studied with respect to cell numbers and cell function. Ethanol administration resulted in the loss of lymphocytes from the peripheral blood, spleen and thymus regardless of the route of ethanol administration (intubation or inhalation in rats, liquid diet for mice). Intubation and liquid diet resulted in a decrease in the ability of lymphocytes from the spleen and peripheral blood to respond to nonspecific mitogens while no such decrease in proliferative ability was noted in rats administered ethanol by inhalation. Adrenalectomy reversed the decrease in ability of lymphocytes to respond to mitogens. The ability to mount a primary immune response was tested using SRBC and TNP-ficol immunization. Animals treated with ethanol showed a decreased ability to respond to the T-cell dependent antigen SRBC but no change in their ability to respond to the T-cell independent antigen TNP-ficol when compared to controls. The ability to mount a primary immune response to SRBC was not reversed by adrenalectomy. IL2 production was monitored during ethanol administration and was found to be increased in comparison to control at the same time that proliferation was decreased. IL2 receptor numbers also appear to be increased in ethanol treated animals. Studies were performed to examine the link between the immune and central nervous systems using IL2 binding to brain tissue as a model.

Project Description:Investigators:

C. Marietta	Physiologist	LPPS, NIAAA
F. Weight	Section Chief	LPPS, NIAAA
T. Jerrells	Immunologist	DRD, WRAIR
D. Peritt	Research Assistant	DRD, WRAIR
W. Smith	Medical Fellow	DRD, WRAIR
M. Eckardt	Section Chief	LCS, NIAAA
A. Parfitt	Biochemist	LPPS, NIAAA

Objectives:

The objectives of this study are: (1) to determine the effects of alcohol on the immune system in an animal model of alcohol dependence that is controlled and well-defined; (2) to determine the mechanism by which the effects occur; (3) to compare the effects of alcohol on the immune system using different animal models of alcohol dependence; and (4) to investigate hypothesized means of communication between the brain and immune systems.

Methods Employed:

Male Sprague-Dawley or Lewis rats were made dependent upon ethanol using the intubation technique of Majchrowicz (Psychopharmacologia 43: 245-254, 1975). Rats were sacrificed daily during the period of intubation and their spleens and thymi removed, and peripheral blood samples collected and analyzed. Rats were also studied 1, 3, 5 and 7 days following the termination of ethanol treatment. Thymus glands and spleens were disaggregated and the number of lymphocytes determined. Peripheral blood differentials were determined and lymphocytes isolated for further study. Lymphocytes from the spleen and peripheral blood were cultured in the presence of nonspecific T-cell (Con A and PHA) and B-cell (STM) mitogens and the ability of the lymphocytes to respond to the mitogens was tested in a  $^3\text{H}$ -thymidine incorporation test. Production of the lymphokine interleukin-2 (IL2) in response to Con A stimulation was also examined. Serum corticosterone levels were determined by RIA. Adrenalectomized and non-adrenalectomized rats were immunized before the start of ethanol treatment as well as at various times during treatment with sheep red blood cells (SRBC) or TNP-ficol. Spleens were obtained at various times after ethanol treatment, disaggregated, counted and incubated with SRBC or TNP-ficol labeled SRBC and complement in a Cunningham chamber for determination of plaque forming cells (a measure of B-cell activity). Similar experiments were performed on rats exposed to ethanol for 14 days using an inhalation chamber. Studies on ethanol's effect on the immune system of C57BL/6 mice were conducted using a liquid diet model of ethanol dependence. Similar studies were performed on spleen cells from mice. Bone marrow cells from ethanol treated rats were cultured in methylcellulose for 2 or 7 days in the presence of saturating concentrations of either erythropoietin or colony-stimulating factor. Colonies were scored as CFU-E or CFU-GM under appropriate conditions. Assays of IL2 binding to Con A stimulated lymphocytes were performed to determine the effect of ethanol on the IL2 receptor. Studies aimed at identifying and localizing IL2 receptors in the brain and determining the effects of ethanol on these IL2 receptors were conducted. Preliminary experiments using radiolabelled receptor binding techniques were performed. Preliminary fluorescence studies are in progress using FACS analysis.

Major Findings:

Spleen, thymus and peripheral blood lymphocyte numbers decreased after treatment with ethanol in all models studied (intubation or inhalation of rats and liquid diet in mice). Spleen and peripheral blood lymphocytes from intubated rats and mice showed a decreased ability to respond to Con A, PHA, or STM while rats exposed to ethanol by inhalation did not show a decrease in ability to respond to nonspecific mitogens. Cell counts and proliferative ability returned toward control levels within 7 days after cessation of ethanol treatment in the intubated rats.

The ability of the rats to respond to an immunization with SRBC (a T-cell dependent antigen) also decreased during ethanol administration in intubated rats and mice fed ethanol-containing liquid diet, but the ability of the rats and mice to respond to a T-cell independent antigen (TNP-ficol) was unaffected by ethanol treatment.

Corticosterone levels were measured because of indications in the literature that increased corticosteroid levels could cause a decrease in lymphocytes, especially in the thymus. Corticosterone levels were measured in the intubated rats and in mice fed a liquid diet containing ethanol. Corticosterone levels varied throughout the experiment with intubated rats in both the ethanol-treated and control rats. Both ethanol-treated and control rats showed a peak of corticosterone 2 days after beginning intubation, although the ethanol-treated group was significantly elevated ( $p < 0.01$ ). The ethanol-treated rats showed another peak of corticosterone during withdrawal which was not seen in the control group. Elevated corticosterone levels have been demonstrated in the mice by previous investigators (Tabakoff et al., J Pharm Pharmac 30: 371-374, 1978).

Adrenalectomy reversed some of the effects of ethanol on the immune system in intubated rats. Defects in lymphocyte proliferation to mitogenic stimuli (Con A, PHA, STM) were reversed by adrenalectomy as was the ethanol induced increase in production of corticosterone. The loss of lymphocytes from the spleen and thymus accompanying ethanol was only partially reversed by adrenalectomy. No effects of adrenalectomy on the inability of rats to mount a primary immune response to SRBC was noted. Experiments using adrenalectomized mice are currently in progress.

Examination of the bone marrow from intubated rats or rats exposed to ethanol by inhalation revealed decreased cellularity and a significant lowering of colony growth in the ethanol treated rats compared to the control rats. The red cell progenitors (CFU-E) were preferentially affected, while the myeloid progenitors (CFU-GM) appeared to be relatively resistant to the effects of ethanol.

IL2 (interleukin 2) is necessary for lymphocytes to respond to a mitogen by proliferation. The administration of ethanol to rats by intubation results in an increase in IL2 production when compared to control at the same time that the ability of lymphocytes to respond to mitogens is at the lowest. Preliminary data on the IL2 receptors indicate an increase in number of receptors on lymphocytes from ethanol treated rats. More experiments are needed to determine if there are changes in receptor affinity with ethanol treatment. IL2 studies to date have been performed on rats treated with ethanol by intubation.

Communication between the central nervous system and the immune system has been inferred from several studies involving the effect of stress on the immune system. One candidate for a chemical messenger between the immune and central nervous

systems is IL2. IL2 is produced by the lymphocyte and there is some indication that glial cells (which are of macrophage lineage) may produce IL2. We have performed binding experiments on whole brain homogenates, purified membrane preparations and synaptosomes. <sup>125</sup>I IL2 was displaced using cold IL2. Problems have been encountered in this project some of which have been the stickiness of IL2, the difficulty in displacing the radiolabel using cold IL2, and the variability from sample to sample probably related to heterogeneity of the tissue with respect to IL2.

Preliminary results of studies using monoclonal antibodies to T-cell subsets of both rats and mice indicate that the T-helper subset appears to be the first subset to be affected by ethanol treatment. T-suppressors and B-cells appear to be less sensitive to the effects of ethanol. Further monoclonal antibody FACS studies will be performed using mouse cells and monoclonal antibodies.

#### Significance to Biomedical Research and the Program of the Institute:

A significant observation of this study has been that the numbers of lymphocytes in the peripheral blood, spleen and thymus decrease regardless of the method of ethanol administration or species examined. The function of the remaining lymphocytes is impaired in ethanol treated animals where the corticosterone level is high, indicating that ethanol's effect on the immune system may be mediated in part by corticosteroids. Because lymphocytes are involved in protecting the body from various infectious diseases, these results provide a basis for investigating the increased risk of alcoholics to infectious diseases.

#### Proposed Course:

Papers are being written and submitted detailing the effects of ethanol administration on the immune system. Further investigations will be aimed at determining the effects of ethanol on the different T-cell subsets.

#### Publications:

Jerrells, T.R., Marietta, C.A., Bone, G., Weight, F.F., and Eckardt, M.J. Effects of ethanol on immune function. Clinical Immunology Newsletter 8: 25-29, 1987.

Jerrells, T.R., Marietta, C.A., Bone, G., Weight, F.F. and Eckardt, M.J. Ethanol-associated immunosuppression. Advances in Biochemical Psychopharmacology, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00478-04 LPPS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ethanol and Drugs of Dependence; Localizing Effects on Brain Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Marietta Physiologist LPPS, NIAAA

Others: M. Eckardt Section Chief LCS, NIAAA  
F. Weight Section Chief LPPS, NIAAA  
G. Szabo Visiting Associate LPPS, NIAAA  
B. Tabakoff Acting Chief LPPS, NIAAA

## COOPERATING UNITS (If any)

Physiology Department, AFRR, Bethesda, MD (K. Zbicz), Dept. Med., Indiana University Medical Center, Indianapolis, IN (T.-K. Li)

## LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

## SECTION

Section on Immunology

## INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.1

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous reports have suggested that localized alterations in brain metabolism occur after administration of ethanol as well as other drugs. General and localized changes in metabolic activity within the brains of rats exposed to various drugs capable of producing physical dependence were studied using the 2-deoxyglucose technique. Specifically, we have studied the chronic administration and subsequent withdrawal as well as the acute effects of ethanol, phenobarbital, diazepam and various peptides on brain glucose metabolism. Similarities and differences were noted in the autoradiographs from rats undergoing withdrawal from ethanol, phenobarbital and diazepam. Among the similarities were the appearance of columns in the frontal sensorimotor cortex and ovoid areas in the cerebellar vermis. The administration of 5 mg/kg of diazepam to an ethanol withdrawing rat resulted in suppression of behavioral withdrawal signs and a loss of columnar and ovoid areas in resulting 2-DG autoradiographs. A general decrease in glucose metabolism was noted in Sprague-Dawley rats given ethanol acutely with an increase in metabolism seen in only the superior olive and dentate region of the hippocampus at a blood ethanol concentration of 14 mM. Differences in glucose metabolism were noted in rats that were bred to prefer or not prefer ethanol. Further experiments and analysis is needed to illustrate a genetic basis for these differences. Experiments are progressing to investigate the effect of various peptides especially peptide T and related peptides) on brain metabolism, using peptide sequences common to the AIDS virus envelope proteins and VIP.

Project Description:Investigators:

C. Marietta	Physiologist	LPPS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
F. Weight	Section Chief	LPPS, NIAAA
K. Zbic	Computer Specialist	DCE, AFRRRI
T.-K. Li	Professor	Dept. Med. Ind. Univ. Med. Center
G. Szabo	Visiting Associate	LPPS, NIAAA
B. Tabakoff	Acting Chief	LPPS, NIAAA

Objectives:

The objectives of this project were: (1) to develop dosing schedules that are successful in inducing physical dependence in laboratory rats to ethanol, phenobarbital and diazepam; (2) to determine the effects of withdrawal from ethanol, phenobarbital and diazepam on brain metabolism; (3) to determine the effects of acute doses of ethanol, phenobarbital and diazepam on brain metabolism; (4) to determine what effect a given dose to diazepam has on the brain metabolism of rats undergoing ethanol withdrawal; (5) to determine if a given dose of ethanol will cause a different pattern of local cerebral glucose utilization in rats bred to either prefer or not prefer alcohol; and (6) to determine the effect of various peptides related to AIDS virus envelope protein on brain metabolism.

Methods Employed:

Sprague-Dawley rats were rendered dependent upon ethanol, phenobarbital or diazepam by oral intubation of the drug in an appropriate vehicle. Control animals were intubated with the vehicle alone. The time course and dosage schedule varied with the drug under study. Drug levels in the blood were monitored throughout each experiment. When withdrawal symptoms were most intense 2-deoxyglucose (2-DG) was used to determine general and localized changes in brain metabolism. Acute effects were determined by the administration of the drug under investigation i.p. 30 min before the i.v. injection of 2-DG. For the studies of the peptides related to AIDS virus envelope protein, intracerebroventricular cannulae were implanted several days prior to the experiment and peptides injected i.c.v. 10 min prior to injection of 2-DG.

Major Findings:

We have found statistically significant general and localized increases in glucose metabolism as measured by 2-[<sup>14</sup>C]-deoxyglucose (2-DG) in rats undergoing withdrawal from ethanol and phenobarbital. Rats undergoing withdrawal from diazepam show statistically significant increases and decreases in glucose utilization. Among similarities noted in withdrawing rats was the appearance of 400 um wide columns in the sensorimotor cortex, ovoid areas in the cerebellar vermis and increases in several nuclei associated with the motor system. This is consistent with the preponderance of withdrawal signs that reflect motor functioning, such as hyperactivity, tremors, and spontaneous convulsions. Differences among the withdrawals included differential increases in the lateral geniculate in diazepam and phenobarbital withdrawals but not ethanol withdrawal.

Acute administration of ethanol produced decreases in glucose metabolism in 20%

of the regions examined. Ethanol doses of 0.8, 1.6 and 3.2 g/kg were examined. These doses gave peak blood ethanol levels of 14, 26 and 66 mM, respectively. Increased metabolism was noted in the dentate region of the hippocampus and the superior olivaris nucleus at 14 mM ethanol. Decreased metabolism was most dramatic in the median raphe, vestibular, cerebellar vermis and various structures associated with the auditory system.

Acute administration of phenobarbital (100 mg/kg) produced autoradiographs indicative of a decrease in 2-DG uptake. This result is similar to other experiments in the literature that indicate a decrease in cerebral  $O_2$  consumption with administration of phenobarbital.

Diazepam (5 mg/kg) given to ethanol-withdrawing rats yielded autoradiographs in which the columns were no longer visible. A general decrease in metabolism throughout the brain was noted when compared to ethanol-withdrawing rats. Metabolic rates were similar to those noted in control rats. These results correlate with an absence of behavioral withdrawal signs in diazepam-treated ethanol-withdrawing rats, indicating that the effect of diazepam on brain glucose utilization during ethanol withdrawal correlates with its effects on the behavioral manifestation of ethanol withdrawal.

The acute administration of diazepam (5 mg/kg) results in decreased glucose utilization throughout the brain. Further statistical analysis is necessary to determine if there are localized areas of decreases as well as the general decrease in glucose utilization in rats treated acutely with diazepam.

The administration of 0.5 g/kg ethanol to outbred and selectively bred ethanol preferring and ethanol non-preferring rats resulted in differences between groups in approximately 47% of the structures examined. Acute ethanol administration was associated with decreased cerebral metabolism in the outbred and ethanol preferring groups and with increased metabolism in the ethanol non-preferring group. Almost no differences were noted between outbred and preferring groups while non-preferring rats had higher cerebral metabolic rates. Additional rats need to be run in order for further analysis to be performed.

Preliminary analysis of autoradiographs from rats injected intracerebroventricularly with the octapeptide, peptide T, and an analogue of the peptide indicate that both peptides altered local cerebral glucose utilization in rats given injections of the two peptides. Additional experiments using various doses of other related (VIP) active and inactive peptides are necessary to complete this study.

#### Significance to Biomedical Research and the Program of the Institute:

These studies indicate that there are similarities and differences in glucose metabolism in the withdrawal syndrome seen with ethanol, phenobarbital and diazepam. The similarities and differences may be significant with respect to the pathophysiology of the withdrawal mechanism(s). An additional significant observation is that the autoradiographic picture of the brain of rats undergoing ethanol withdrawal but given 5 mg/kg of diazepam correlates with changes in behavior of the rats given diazepam, a drug used to treat withdrawal symptoms in humans. This suggests the possible use of the 2-DG technique in ethanol-withdrawing rats to screen new drugs for the treatment of ethanol withdrawal. The results of the acute studies indicate that differences in glucose metabolism can be seen with low or high doses of ethanol. The strain of animals used also

appears to be important, as noted in the preliminary results from studies dealing with ethanol preferring and non-preferring rats given the same dose of ethanol and compared to outbred rats of the parent strain. The effects of various peptides on brain glucose metabolism could lead to an understanding of how normal brain metabolism can be affected by foreign peptides.

Proposed Course:

Continued statistical and densitometric analysis will be performed on these projects. Further investigations on the effects of a given dose of ethanol on ethanol preferring and ethanol non-preferring rats, as well as the effects of icv injections of active and inactive peptides will continue. Several papers are in various stages of preparation for publication.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00700-03 LPPS
PERIOD COVERED October 1, 1986 - September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Ethanol Effects on Membrane-Bound Enzymes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI:        P. Hoffman                      Section Chief                      LPPS, NIAAA B. Tabakoff                    Acting Chief                        LPPS, NIAAA		
Others:    P.T. Nhamburo                    Visiting Fellow                    LPPS, NIAAA P. Rathna Giri                   Visiting Associate                LPPS, NIAAA G. Szabo                            Visiting Associate                LPPS, NIAAA		
COOPERATING UNITS (if any) University of Illinois Coll. Med. Rockford (B. Salafsky); University of Illinois, Chicago (J. M. Lee); Westside VA, Chicago (F. De Leon-Jones); Sapporo Medical College (T. Saito); IRP, NINCDS, (G. Eisenhofer).		
LAB/BRANCH Laboratory of Physiologic and Pharmacologic Studies		
SECTION Section on Receptor Mechanisms		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Ave., Rockville, MD 20852		
TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 2.5	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>             It has been hypothesized that the actions of ethanol in the CNS result from its ability to perturb the structure of neuronal membrane lipids. The activities of membrane-bound enzymes, which are modulated by the properties of surrounding lipids, may serve as probes to localize specific sites of action of ethanol within the neuronal membrane. (Na<sup>+</sup>,K<sup>+</sup>)ATPase exists in two forms in brain, with high and low affinity for ouabain. The high-affinity component is thought to be localized in neuronal membranes. High concentrations of ethanol, <u>in vitro</u>, selectively inhibited the activity and increased ouabain sensitivity of the high-affinity form of the enzyme. After chronic, <u>in vivo</u> ingestion of ethanol, producing tolerance and physical dependence, the sensitivity to ouabain of the neuronal form of the enzyme was selectively increased, and this form of the enzyme was resistant to the <u>in vitro</u> effect of ethanol. Monoamine oxidase (MAO) in brain also exists in two forms, A and B, and we previously showed that ethanol selectively inhibits the B form of MAO, which is also found in platelets. We have found that inhibition of platelet MAO by ethanol is increased in platelets of alcoholics compared to controls. The activity of platelet adenylate cyclase, however, is lower in platelets of alcoholics. These two activities may be useful as markers for alcohol consumption. The effects of ethanol <u>in vivo</u> on other enzymes of neurotransmitter metabolism are being studied. Using an irreversible inhibitor of DOPA decarboxylase, monofluoromethyl-dopa, we have found that catecholamine metabolism in several brain areas and in peripheral organs is increased after chronic ethanol ingestion. Purification of the brain enzyme, succinic semialdehyde reductase, which may be involved in GABA metabolism, has been initiated. These studies add to our understanding of the regulation of neuronal activity, as reflected by neurotransmitter turnover, and the effects of ethanol on that activity.           </p>		

Project DescriptionInvestigators

P. Hoffman	Section Chief	LPPS, NIAAA
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Objectives:

The goal of this project is to characterize acute (*in vitro*) and chronic effects of ethanol on the activity of CNS enzymes involved in neurotransmission. Many of these enzymes are membrane-bound, and their activity is modified by the properties of the lipids that surround them. As a result, the enzyme activities can serve as specific probes to elucidate the site of action of ethanol within the neuronal membrane. Similarly, changes in enzyme activity following chronic, *in vivo*, ethanol ingestion may reflect alterations in the properties of selected areas of neuronal membranes. Such changes may be associated with neuroadaptive responses (tolerance, physical dependence) to ethanol. The studies of the effects of ethanol on (Na<sup>+</sup>,K<sup>+</sup>)ATPase activities were designed to evaluate the specificity of action of ethanol, as well as the contributions of lipid and protein moieties within the membrane to the action of ethanol. To further elucidate specific sites and mechanisms of action of ethanol in the CNS, enzymes involved in the regulation of neurotransmitter metabolism are being studied, and the effects of ethanol *in vivo* on the activity of enzymes of neurotransmitter metabolism---which reflect neuronal activities---are being evaluated. Diagnostic tools that would be of value to clinicians include objective indices of chronic ethanol consumption, as well as biochemical markers that might identify individuals at risk for developing alcoholism. Previous work with monoamine oxidase (MAO; Tabakoff et al., *Psychopharmacol.* 87: 152, 1985) and adenylate cyclase (Saito et al., *J. Neurochem.* 44: 1037, 1985) activities suggested that these enzyme activities might represent such diagnostic tools. A further objective of this project was to evaluate these enzyme activities in platelets of alcoholic and control subjects.

Methods:

**ETHANOL TREATMENT.** For chronic ethanol treatment, male C57BL/6NCR mice (23-25g) were individually housed and fed a liquid diet containing ethanol or an equicaloric amount of sucrose (controls) for seven days (Ritzmann and Tabakoff, *J. Pharmacol. Exptl. Ther.* 199: 158, 1976). The amount of

diet offered to the control animals was adjusted daily to match the amount consumed by the ethanol-fed mice. This treatment regimen produced functional tolerance to and physical dependence on ethanol.

( $\text{Na}^+$ , $\text{K}^+$ )ATPase ACTIVITY. Mice were sacrificed by decapitation and a crude synaptosomal preparation of cerebral cortex was prepared. ( $\text{Na}^+$ , $\text{K}^+$ )ATPase activity was determined as described by Atterwill et al. (J. Neurochem. 43: 8, 1984), using gamma-32P-ATP. Tissue was preincubated with varying concentrations of ouabain, and data were analyzed by means of non-linear weighted least squares minimization using the program PENNZYME.

MAO ACTIVITY. MAO activity and inhibition of activity by ethanol was measured in human platelets. Platelets were prepared from whole blood which was obtained by venipuncture from alcoholic and control individuals who had given informed consent. The platelets were lysed in hypotonic buffer, frozen and thawed to provide membranes for enzyme assays. MAO activity was assayed by a modification of the spectrophotometric method of Tabakoff and Alivisatos (Anal. Chem. 44: 427, 1972), using dimethylaminobenzylamine (DAB) as substrate, and also by a modification of a radioisotopic assay using 14C-phenylethylamine (PEA) as substrate (Wurtman and Axelrod, Biochem. Pharmacol. 12: 1439, 1963).

ADENYLATE CYCLASE ACTIVITY. Platelets were obtained from whole blood, and platelet membranes (30,000xg pellet) were prepared as described above. Adenylate cyclase activity was assayed by determining the conversion of 32P-ATP to 32P-cAMP by previously described methods (Saito et al., J. Neurochem. 44: 1037, 1985).

BRAIN CATECHOLAMINE TURNOVER. Male C57BL mice were fed ethanol in a liquid diet for seven days, as described above. At the time of withdrawal, they were injected ip with DL-alpha-monofluoromethylidopa, and were sacrificed at various times after injection. Brains were removed and cortex, hippocampus, striatum, brainstem and cerebellum were dissected and homogenized in 0.4N PCA. Brain amine and metabolite levels were analyzed by HPLC to determine rates of catecholamine turnover.

SUCCINIC SEMIALDEHYDE REDUCTASE. Succinic semialdehyde (SSA) reductase was partially purified from liver and brain by ammonium sulfate precipitation and column chromatography on CM cellulose and DEAE cellulose (see Hoffman et al., J. Neurochem. 35: 354, 1980). The enzyme activity was further purified on affinity columns of Blue Sepharose and AG-ADP. Enzyme activity in the presence of NADPH and SSA was assayed by monitoring the change in absorbance at 340 nM.

### Major Findings:

( $\text{Na}^+$ , $\text{K}^+$ )ATPase in brain has been demonstrated to exist in two forms, with different affinity for the inhibitor, ouabain. The form of the enzyme with high affinity for ouabain is thought to be localized neuronally. In our studies, plots of ouabain inhibition of mouse brain ( $\text{Na}^+$ , $\text{K}^+$ )ATPase activity fit a two-site model. In vitro, high concentrations of ethanol selectively reduced the activity, and increased the sensitivity to ouabain inhibition, of the form of the enzyme with high affinity for ouabain. Changes in this fraction of enzyme activity were also found in mice that were fed ethanol chronically and were tolerant to and physically dependent on ethanol. In these animals, the sensitivity to ouabain of the neuronally-localized form of the enzyme was selectively increased, while there was no change in activity or proportion of the two forms of the enzyme. The

neuronally-localized form of the enzyme was resistant to the in vitro effects of ethanol.

The mechanism for the acute, in vitro effects of ethanol and for the change in sensitivity to ouabain after chronic ethanol ingestion may involve changes in the properties of lipids immediately surrounding the ATPase, or effects on the enzyme protein per se. Neither the acute effects of ethanol, which occurred only at high concentrations, nor the changes in ouabain sensitivity after chronic ethanol ingestion would be expected to significantly alter (Na<sup>+</sup>,K<sup>+</sup>)ATPase activity in vivo. The data, however, clearly demonstrate the specificity of the site of action of ethanol in the CNS, since only a single form of two closely-related enzyme activities was affected by ethanol.

Another enzyme which is inhibited by ethanol is monoamine oxidase (MAO). MAO also exists in two forms with differing substrate specificities, known as MAO-A and MAO-B. Platelet MAO has the characteristics of MAO-B, and ethanol was previously found to selectively inhibit the B-form of MAO in human platelets and brain (Tabakoff et al., *Psychopharmacol.* 87: 152, 1985).

We have now found that ethanol inhibition of MAO activity is increased in platelet membranes of male alcoholics, as compared to controls matched for age, sex and socioeconomic status. In contrast to some earlier reports, no difference in platelet MAO activity per se was found between alcoholics and controls. In earlier animal studies, we found that ethanol acutely stimulated brain adenylate cyclase (AC) activity, and that receptor-stimulated AC activity was reduced in brains of mice that had ingested ethanol chronically (Luthin and Tabakoff, *J. Pharmacol. Exptl. Ther.* 228: 579, 1984; Tabakoff and Hoffman, *J. Pharmacol. Exptl. Ther.* 208: 216, 1979). This enzyme activity in platelets might therefore represent an indicator of ethanol consumption. We found that basal AC activity did not differ between controls and alcoholics, but stimulation of AC activity by various agents (e.g., fluoride) was reduced in platelets of alcoholics. The differences in platelet enzyme activities were not associated with age, race, smoking or illicit drug use, and there was no significant correlation with duration of problems with alcohol. The changes were long-lasting, with lower fluoride-stimulated AC activity observed in alcoholic subjects who had abstained from alcohol for 1-4 years. Discriminant analysis showed that the use of the two platelet enzyme assays could correctly classify 75% of alcoholics and 73% of controls. These differences in enzyme activities could represent inherent characteristics of alcoholics, or could be a result of chronic ethanol ingestion.

Studies of catecholamine turnover in mouse brain and peripheral organs are important for evaluating the sites of action of ethanol in the CNS, and for determining whether the effects of ethanol on neurotransmitter receptor characteristics are direct or indirect. The use of monofluoromethyl dopa, an irreversible inhibitor of DOPA decarboxylase, allows measurement both of the rate of L-DOPA accumulation, and the rates of norepinephrine and dopamine depletion (Palfreyman et al., *J. Pharmacol. Meth.* 11: 239, 1984). These data can provide a determination of the contribution of changes in dopamine and norepinephrine metabolism to alterations in DOPA levels, and permit a finer analysis than was previously possible of the effects of ethanol on catecholamine metabolism, which reflects the activity of catecholaminergic neurons. Preliminary results indicate the feasibility of using this method in mouse brain, and demonstrate that chronic ethanol ingestion results in increased rates of DOPA accumulation in hippocampus, cortex and brainstem, measured at the time of ethanol withdrawal.



Purification of SSA reductase, which may be involved in GABA metabolism, has been initiated. The enzyme is a dimer, and can be distinguished from other aldehyde reductases in brain. Its monomer molecular weight, estimated from SDS-PAGE, is 55kDa.

#### Significance to Biomedical Research and the Program of the Institute:

The finding that characteristics of platelet enzymes differ in alcoholics and controls has the potential to provide a simple and reliable diagnostic tool to identify individuals who have consumed ethanol chronically, or who have a genetic predisposition to problems with alcohol. Such objective, easily-assayable measures would be of value to clinicians dealing with alcoholic patients. These findings also provide a model system in which to investigate the mechanism by which ethanol may produce alterations in the activity of specific membrane-bound enzymes.

The selective alterations in the sensitivity to ouabain of neuronal (Na<sup>+</sup>,K<sup>+</sup>)ATPase indicate that chronic ethanol treatment results in specifically localized alterations in neuronal membrane properties, which can be probed by the use of enzyme activity, and which may be important in particular aspects of adaptation to the chronic effects of ethanol. These studies support the hypothesis that different forms of membrane-bound enzymes can reflect differences in their membrane lipid microenvironments, and thus provide basic information about enzyme properties.

The studies of the effects of chronic ethanol ingestion on catecholamine turnover provide an opportunity to develop more precise methods for neurochemical evaluation of neuronal activity, and the effects of ethanol on that activity. These investigations will provide insight into the mechanism by which ethanol alters neurotransmitter receptor function, as well as catecholamine turnover.

#### Proposed Course:

The generality of the findings with platelet enzymes of alcoholics will be tested by measuring these activities in platelets obtained from different populations of alcoholics and controls. Studies of ouabain sensitivity of (Na<sup>+</sup>,K<sup>+</sup>)ATPase in erythrocytes of alcoholics will also be initiated, to determine if this enzyme activity can also distinguish alcoholic individuals.

Alterations in catecholamine metabolism in brains of mice ingesting ethanol chronically will be analyzed in detail to determine possible differential effects on dopamine and norepinephrine metabolism in particular brain areas. The time course for development of these changes during ethanol ingestion will be assessed and compared to changes in receptor properties.

Purification of SSA reductase will be pursued, in order to produce antibodies to the enzyme that can be used to localize activity in brain. These studies will contribute to an understanding of the role of the enzyme in GABA metabolism, which may be altered during ethanol ingestion.

Studies of the effects of ethanol on ouabain inhibition of (Na<sup>+</sup>,K<sup>+</sup>)ATPase in brain are complete.

Publications:

1. Tabakoff, B., Hoffman, P.L. and Liljequist, S.: Effects of ethanol on the activity of brain enzymes. Enzyme 37: 70-86, 1987.
2. Nhamburo, P.T., Salafsky, B.P., Tabakoff, B. and Hoffman, P.L.: Effects of ethanol on ouabain inhibition of mouse brain (Na<sup>+</sup>,K<sup>+</sup>)ATPase activity. Biochem. Pharmacol. 36: 2027-2033, 1987.
3. Saito, T., Ozawa, H., Tsuchiya, F., Ishizawa, H. and Tabakoff, B.: Effects of ethanol on adenylate cyclase system in the human platelet. Alcohol and Alcoholism Suppl. 1: 761-765, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00702-03 LPPS
PERIOD COVERED October 1, 1986 - September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Ethanol Modification of Neurotransmitter Receptor-Effector Coupling</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: P. Hoffman                      Section Chief                      LPPS, NIAAA B. Tabakoff                     Acting Chief                      LPPS, NIAAA		
Other: P. Valverius                      Visiting Fellow                      LPPS, NIAAA P. T. Namburo                    Visiting Fellow                      LPPS, NIAAA G. Szabó                            Visiting Associate                   LPPS, NIAAA		
COOPERATING UNITS (if any) NIMH, CNB, (S. Paul, P. Suzdak); Univ. of Illinois Med. Ctr., (J.M. Lee); Sapporo Med. College, (T. Saito); Univ. of Illinois Coll. Med. Rockford (B. Salafsky, S. Khatami)		
LAB/BRANCH Laboratory of Physiologic and Pharmacologic Studies		
SECTION Section on Receptor Mechanisms		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Ave., Rockville, MD 20852		
TOTAL MAN-YEARS: 5.5	PROFESSIONAL: 4.5	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Ethanol selectively alters the function of neurotransmitter and neuromodulator receptors in the CNS. These changes may contribute to the acute, intoxicating effects of ethanol, and adaptations in receptor function may be associated with ethanol tolerance and/or physical dependence. The imidazobenzodiazepine, Ro15-4513, a putative ethanol antagonist, was used to assess the role of the GABA/benzodiazepine receptor in the acute effects of ethanol. Ro15-4513 reduced the incoordinating, but not the hypothermic effect of ethanol in mice, indicating that certain of ethanol's actions may involve the GABA receptor complex. Biochemical and behavioral methods were used to determine the role of receptor-coupled adenylate cyclase (AC) systems in ethanol's effects. In mouse cerebral cortex, low concentrations of ethanol in vitro stimulated isoproterenol-sensitive AC activity and altered high-affinity agonist binding. The data suggested that ethanol affected Gs, the guanine nucleotide binding protein. In ethanol tolerant/dependent animals, high-affinity agonist binding was undetectable, and stimulation of AC activity by isoproterenol and other agonists was reduced, reminiscent of changes that occur during heterologous desensitization, and that may involve a change in G-protein function. Analysis of cortical G-proteins in these animals by cholera toxin and pertussis toxin-induced 32P-ADP ribosylation and SDS-PAGE revealed a selective decrease in the amount of Gs. The potential importance of adenylate cyclase activity in neuroadaptation to ethanol was suggested by the finding that administration of forskolin, which stimulates brain AC activity, overcame the blockade of tolerance development produced by norepinephrine depletion. Chronic ethanol ingestion produced different changes in beta-adrenergic receptor characteristics in cerebellum (primarily beta-2 receptors) than in cortex and hippocampus (primarily beta-1), suggesting that ethanol affects the coupling of these receptor subtypes, and/or regional G-protein characteristics, in a selective manner. Chronic ethanol ingestion also selectively reduced the number of mu opiate receptors in cerebral cortex.		

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S. Paul	Chief	CNB, NIMH
P. Suzdak	Staff Fellow	CNB, NIMH
B. Salafsky	Professor	Univ. of IL Coll. Med. Rockford

Objectives:

The goal of this project is to determine the effects of ethanol on neurotransmitter receptor-effector coupling processes. It has been postulated that the acute effects of ethanol (e.g., intoxication), as well as adaptation to these effects (tolerance, physical dependence) are mediated by changes in neuronal membrane lipid properties. However, the functional moieties within the membranes are proteins, such as receptors. Our previous work showed that ethanol has specific sites of action within the receptor-adenylate cyclase (AC) systems in brain, selectively affecting the protein-protein interactions that mediate receptor-effector coupling processes. Our objective in this project continues to be a determination of the specificity and mechanism of action of ethanol on receptor-coupled AC systems, and other receptor-effector systems, and the role of these systems in neuroadaptation to ethanol. These studies define the sites of action of ethanol within the CNS, and determine if adaptive changes in the receptor-effector systems occur at the initial sites of action of ethanol, and may be associated with specific behavioral aspects of ethanol tolerance or physical dependence.

Methods:

ETHANOL AND OTHER DRUG TREATMENTS. For chronic ethanol treatment, male C57BL mice (23-25g) were individually housed and fed a liquid diet containing ethanol or an equicaloric amount of sucrose (controls) for seven days (Ritzmann and Tabakoff, J. Pharmacol. Exptl. Ther. 199: 158, 1976). The amount of diet offered to the control animals was adjusted daily to match the amount consumed by the ethanol-fed mice. This treatment regimen produced functional tolerance to and physical dependence on ethanol. Animals were sacrificed either at the time of withdrawal or at various times after withdrawal. Tolerance was tested, and withdrawal symptoms were monitored, in ethanol-fed mice that were not used for biochemical studies. The incoordinating, hypnotic and hypothermic effects of ethanol were measured as described previously (Ritzmann and Tabakoff, J. Pharmacol. Exptl. Ther. 199: 158, 1976; Hoffman and Tabakoff, Pharmacol. Biochem. Behav. 21: 539, 1984). Ro15-4513

was administered intraperitoneally (ip) prior to ethanol injection (ip). Forskolin, 6-OHDA or the phorbol ester, phorbol 12-myristate, 13-acetate, were administered intracerebroventricularly (icv) through previously implanted cannulae. Forskolin and the phorbol ester were administered during the time that mice were ingesting the liquid diets; 6-OHDA was administered one week prior to initiation of the liquid diet regimen.

**ADENYLATE CYCLASE ASSAY.** Animals were killed by decapitation, brains were removed, and a 48,000xg membrane fraction was prepared as previously described (Luthin and Tabakoff, J. Pharmacol. Exptl. Ther. 228: 579, 1984). Adenylate cyclase activity was measured by determining the production of 32P-cyclic AMP from 32P-ATP (Tabakoff and Hoffman, J. Pharmacol. Exptl. Ther. 208: 216, 1979).

**BETA-ADRENERGIC RECEPTOR BINDING ASSAY.** Animals were killed by decapitation, and brains were rapidly removed. Cerebral cortical, hippocampal and cerebellar membranes (48,000xg fraction) were prepared as described above. Antagonist (125I-iodocyanopindolol; ICYP) binding was assayed according to the method of Engel et al. (N-S. Arch. Pharmacol. 317: 277, 1981). Agonist (isoproterenol; ISO) binding was assayed by displacement of bound ICYP. Data were analyzed using the LIGAND program (Munson and Rodbard, Anal. Biochem. 107: 220, 1980).

**OPIATE RECEPTOR BINDING ASSAY.** Mice were killed by decapitation, and membrane fractions were prepared as described above. Opiate binding to receptor subtypes was assayed using 3H-2-D-Ala-MePhe-glyol-enkephalin, 3H-2-D-Ala,5-D-Leu-enkephalin or 3H-bremazocine, in the presence of high concentrations of masking ligands, where necessary, by methods described previously (Gilliam et al., Br. J. Pharmacol. 70: 481, 1980; Pasternak et al., Mol. Pharmacol. 11: 340, 1975; Tabakoff and Hoffman, Life Sci. 32: 197, 1983).

**QUANTITATION OF GUANINE NUCLEOTIDE BINDING PROTEINS.** Mice were sacrificed by decapitation and cerebral cortical membranes, prepared as described above, were incubated with alpha-32P-NAD<sup>+</sup> in the presence or absence of activated cholera toxin or pertussis toxin (Ribiero-Neto et al., Meth. Enzymol. 109: 566, 1985). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, Nature, New Biol. 227: 680, 1970) and 32P-ADP-ribose incorporation was determined by autoradiography.

### Major Findings:

The results obtained elucidate the role of particular neurotransmitter and neuromodulator receptors and receptor-effector coupling processes in the acute effects of ethanol, and in neuroadaptive responses of the CNS to ethanol (tolerance and physical dependence). The imidazobenzodiazepine, Ro15-4513, a partial inverse agonist at brain benzodiazepine receptors, was previously suggested to represent an "ethanol antagonist" in rats (Suzdak et al., Science 234: 1243, 1986). We found that Ro15-4513, administered to mice after ethanol, did reduce the incoordinating effect of ethanol, measured on an accelerating Rotarod apparatus. This effect of Ro15-4513 was reduced by a benzodiazepine receptor antagonist. In contrast, Ro15-4513 did not alter the hypothermic response to ethanol. However, Ro15-4513 reduced both the incoordinating and hypothermic effects of pentobarbital in mice. These data support the postulate that Ro15-4513 can antagonize effects of ethanol, as well as effects of other drugs, such as pentobarbital, that are mediated by an action at the GABA-benzodiazepine receptor-coupled chloride channel. That is, the incoordinating effect of ethanol, as well as its sedative effects, may result from

ethanol-induced modulation of the function of this ion channel. However, other effects of ethanol, including its hypothermic effect, appear to be mediated by other CNS neuronal systems. Ro15-4513 does not block all the effects of ethanol, but, by altering the ability of ethanol to affect the function of the GABA-benzodiazepine receptor complex, selectively affects certain responses to ethanol. Understanding the antagonistic effect of Ro15-4513 may facilitate the development of therapies to reduce certain behavioral effects of ethanol. Mice that are pretreated with 6-OHDA, to partially destroy brain noradrenergic systems, do not develop tolerance to ethanol when they are fed ethanol chronically in a liquid diet (Tabakoff and Ritzmann, *J. Pharmacol. Exptl. Ther.* **203**: 319, 1977), in contrast to vehicle-treated mice. We have found that daily treatment of mice with forskolin during the time that they are ingesting ethanol allows the 6-OHDA-treated mice to develop tolerance to ethanol. Forskolin is a potent activator of adenylate cyclase, and presumably increases the level of cAMP in mouse brain, similar to the action of norepinephrine at beta-adrenergic receptors. Norepinephrine, acting at alpha-1 adrenergic receptors, stimulates phosphatidylinositol metabolism and, through increases in intracellular calcium levels and diacylglycerol production, activates protein kinase C. However, norepinephrine-depleted mice treated with phorbol 12-myristate, 13-acetate, an activator of protein kinase C, did not develop ethanol tolerance. These data imply an important role of adenylate cyclase activity in neuroadaptation to ethanol, specifically, in development of functional ethanol tolerance. Our biochemical data also support a role for this receptor-effector coupling system in the response to ethanol. We found that ethanol *in vitro*, which stimulates adenylate cyclase activity (Saito et al., *J. Neurochem.* **44**: 1037, 1985), specifically altered high-affinity binding of isoproterenol to cerebral cortical membranes. High-affinity agonist binding is thought to reflect the formation of an agonist-receptor-G-protein complex, and ethanol appeared to interact with Gs to alter affinity for agonist. After chronic ethanol ingestion, high-affinity isoproterenol binding was no longer detectable, suggesting an uncoupling of receptor and G-protein, compatible with the decreased stimulation of adenylate cyclase by isoproterenol that is also observed in the ethanol-fed animals. The nature of the changes in beta-adrenergic receptor function was similar to that seen during heterologous desensitization. Furthermore, we found that, in cortical membranes of ethanol-fed animals, adenylate cyclase activity was less responsive to adenosine, vasoactive intestinal peptide (VIP) and forskolin, all of which act (at least in part) via Gs. Changes in cerebral cortical G-proteins were assessed by measuring cholera and pertussis toxin-induced labeling with 32P-ADP-ribose, and separating the proteins on SDS-PAGE. In membranes from mice treated chronically with ethanol, there was a selective decrease in the amount of labeled Gs, with no detectable change in Gi. This decrease could contribute both to the lack of high-affinity binding of beta-adrenergic agonist, and the decreased response of adenylate cyclase to stimulation by several agonists in ethanol-fed mice. Whether there is an actual decrease in the quantity of Gs, or a change in Gs structure that results in decreased labeling, remains to be determined. As described above, it appears that stimulation of adenylate cyclase activity may be important for the development of tolerance to ethanol. The role of specific receptor-adenylate cyclase systems, other than the beta-adrenergic system, needs further elucidation. Furthermore, we have found that chronic ethanol ingestion has a different effect on cerebellar beta-adrenergic receptors (beta-2 receptors) than on cortical and hippocampal beta-adrenergic receptors (beta-1 receptors). In cerebellum, the number of receptors is reduced, but both high- and low-affinity agonist binding sites are detectable at the time of ethanol withdrawal. This change is similar to that which occurs during homologous desensitization, and may reflect an alteration

in the specific receptor, rather than a change in G-protein amount or function. Thus, the effects of ethanol appear to vary somewhat within different brain regions.

We have also found a selective effect of chronic ethanol ingestion on opiate receptor subtypes. In mouse frontal cortex, there was a decrease in mu opiate receptor number with no change in delta or kappa receptors. This change does not appear to be an adaptation to the acute effects of ethanol on opiate receptor function, and may represent a pathological alteration that can contribute to certain specific CNS effects of ethanol, including alterations in the regulation of mesolimbic dopaminergic function.

#### Significance to Biomedical Research and the Program of the Institute:

Ethanol is generally considered to be a drug that does not act at a specific receptor, and a hypothesis to explain the CNS effects of ethanol is based on its ability to perturb neuronal membrane lipid structure in a relatively non-specific manner. In contrast, our data demonstrate that ethanol has specific sites of action within receptor-effector coupling processes, or at receptor sites per se. In particular, receptor-coupled adenylate cyclase systems appear to be sensitive to effects of ethanol. Furthermore, our data suggest that changes in these systems may be important for neuroadaptation to ethanol. Changes in the function of these ubiquitous receptor-coupled AC systems as a result of chronic ingestion of ethanol could produce neurochemical imbalances that may contribute to specific signs of ethanol tolerance and/or physical dependence, and suggest means for modulating these adaptive processes. These studies also provide insight into basic mechanisms of receptor-effector coupling, and the physiological adaptive responses of these systems.

The behavioral studies described are important for an understanding both of the acute effects of ethanol, and of the development of ethanol tolerance. In particular, the studies with Ro15-4513 not only lead to a more detailed understanding of the site and mechanism of ethanol's action, but may contribute to the development of therapies to counter particular responses to ethanol.

#### Proposed Course:

Changes in brain G-proteins (Gs, Gi, Go), including quantitative and qualitative changes that occur after chronic ethanol ingestion, and the molecular mechanisms of these changes, will be investigated in several brain areas. The relationship of the changes to ethanol tolerance and physical dependence will be examined. The role of second messenger systems in the development of ethanol tolerance (tolerance to several effects of ethanol) or dependence will be further elucidated using compounds that alter brain cyclic AMP levels.

#### Publications:

1. Liljequist, S. and Tabakoff, B.: Bicuculline-pentobarbital interactions on [35S]TBPS binding in various brain areas. Life Sci. 39: 851-855, 1986.
2. Garret, K. M. and Tabakoff, B.: Effects of prenatal phenobarbital on benzodiazepine receptor development. J. Neurochem. 47: 1154-1160, 1986.

3. Saito, T., Lee, J.M., Hoffman, P.L. and Tabakoff, B.: Effects of chronic ethanol treatment on the beta-adrenergic receptor-coupled adenylate cyclase system of mouse cerebral cortex. J. Neurochem. 48: 1817-1822, 1987.
4. Hoffman, P.L., Tabakoff, B., Szabó, G., Suzdak, P. and Paul, S.M.: Effect of an imidazobenzodiazepine, Ro15-4513, on the incoordination and hypothermia produced by ethanol and pentobarbital. Life Sci. 41: 611-619, 1987.
5. Liljequist, A., Culp, S. and Tabakoff, B.: Ethanol-induced modulation of 35S-TBPS binding. Alcohol and Alcoholism Suppl. 1: 653-656, 1987.
6. Hoffman, P.L., Valverius, P., Kwast, M. and Tabakoff, B.: Comparison of the effects of ethanol on beta-adrenergic receptors in heart and brain. Alcohol and Alcoholism Suppl. 1: 749-754, 1987.
7. Saito, T., Luthin, G.R., Lee, J.M., Hoffman, P.L. and Tabakoff, B.: Differential effects of ethanol on the striatal and cortical adenylate cyclase system. Jap. J. Pharmacol. 43: 133-141, 1987.
8. Hoffman, P.L., Saito, T. and Tabakoff, B.: Selective effects of ethanol on neurotransmitter receptor-effector coupling systems in brain. Ann. N.Y. Acad. Sci. 492: 396-398, 1987.
9. Tabakoff, B. and Hoffman, P.L.: Interaction of ethanol with opiate receptors: implications for the mechanism of action of ethanol. In Engel, J. and Oreland L. (Eds.): Brain Reward Systems and Abuse. New York, Raven Press, 1987, pp. 99-107.
10. Khatami, S., Hoffman, P.L., Shibuya, T. and Salafsky, B.: Selective effects of ethanol on brain opiate receptor subtypes. Neuropharmacol., in press.
11. Valverius, P., Hoffman, P.L. and Tabakoff, B.: Effect of ethanol on mouse cerebral cortical beta-adrenergic receptors. Mol. Pharmacol., in press.
12. Tabakoff, B., Hoffman, P.L., Liljequist, S., Eckardt, M.J., Marietta, C.A., Majchrowicz, E. and Weight, F.F.: Effects of chronic ethanol ingestion on brain metabolism and receptor systems. Proc. Intl. Symp. Med. Biol. Prob. Alcoholism, in press.
13. Tabakoff, B., Luthin, G., Saito, T. and Hoffman, P.L.: Ethanol's actions on receptor-effector coupling in brain. Proc. 5th Intl. Conf. Rec. Adv. Biomed. Aspects Alcohol and Alcoholism, in press.
14. Tabakoff, B. and Hoffman, P.L. Biochemical pharmacology of alcohol. In Meltzer, H., et al. (Eds.): Psychopharmacology: The Third Generation of Progress. New York, Raven Press, in press.



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00703-03 LPPS
PERIOD COVERED October 1, 1986 - September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Neurohypophyseal Peptides and Ethanol Tolerance		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: P. Hoffman      Section Chief      LPPS, NIAAA  Others: B. Tabakoff      Acting Chief      LPPS, NIAAA G. Szabó      Visiting Associate      LPPS, NIAAA L. Liu      Guest Researcher      LPPS, NIAAA J. Dave      Visiting Associate      LPPS, NIAAA S. Culp      Physical Sci. Tech.      LPPS, NIAAA H. Ishizawa      Visiting Fellow      LPPS, NIAAA		
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LAB/BRANCH Laboratory of Physiologic and Pharmacologic Studies		
SECTION Section on Receptor Mechanisms		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Ave., Rockville, MD 20852		
TOTAL MAN-YEARS: 4.5	PROFESSIONAL: 3.5	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Arginine vasopressin (AVP) and related peptides, when administered exogenously, prolong the duration of tolerance to ethanol. The similarities between neurohypophyseal peptide effects on tolerance and previously reported effects on memory consolidation supported the hypothesis that these phenomena may share underlying mechanisms. Using peptide agonists that interact selectively with vasopressin V1 and V2 receptors, we have characterized as V1 the receptors in brain that mediate the ability of vasopressin to maintain ethanol tolerance. A role for endogenous AVP, acting at brain V1 receptors, in the maintenance of tolerance was supported by the finding that a V1-selective antagonist, given alone, enhanced the rate of loss of ethanol tolerance, while a V2-selective antagonist did not. In the periphery, V1 vasopressin receptors mediate peptide-induced alterations in calcium mobilization. The ability of AVP to maintain ethanol tolerance was previously shown to depend on the presence of intact noradrenergic systems in brain, and the interaction of AVP with neuronally localized V1 receptors may alter norepinephrine release, and thus modulate tolerance. Vasopressin, as well as oxytocin and related peptides, were found to block or delay the development of ethanol tolerance. The two actions of AVP may be mediated via different receptors. The role of endogenous hormone in the development and maintenance of ethanol tolerance is also being investigated through studies of the biosynthesis and release of AVP in hypothalamus, posterior pituitary and other tissues (anterior pituitary, testis, and Leydig cell cultures). These studies will investigate mRNA, neurophysin and hormone levels during chronic ethanol treatment leading to the development of tolerance. Preliminary studies indicate that both hypothalamic and extrahypothalamic hormone synthesis are increased by dehydration. Other physiological regulators are also being studied. Understanding the role and mechanism of action of AVP in development, expression and dissipation of tolerance to ethanol may lead to benign means for the manipulation of tolerance and, possibly, of ethanol intake.</p>		

Project DescriptionInvestigators:

P. Hoffman	Section Chief	LPPS, NIAAA
B. Tabakoff	Acting Chief	LPPS, NIAAA
G. Szabo	Visiting Associate	LPPS, NIAAA
L. Liu	Guest Researcher	LPPS, NIAAA
J. Dave	Visiting Associate	LPPS, NIAAA
H. Ishizawa	Visiting Fellow	LPPS, NIAAA
S. Culp	Physical Sci. Tech.	LPPS, NIAAA
M. Brownstein	Chief	LCB, NIMH
S. Young	Staff Fellow	LCB, NIMH

Objectives:

Arginine vasopressin (AVP), a mammalian antidiuretic hormone, and structurally-related peptides, can maintain functional tolerance to ethanol in mice and rats, once that tolerance has been established. The investigations demonstrating this effect of AVP have involved administration of exogenous hormone or its analogs. The goals of this project include characterization of the CNS receptors and neurochemical mechanisms involved in the action of the hormone, evaluation of the role of endogenous hormone in the maintenance of tolerance, and determination of the effects of AVP on development of ethanol tolerance. Further goals include a detailed evaluation of the effects of acute and chronic ethanol administration on the biosynthesis and release of AVP and oxytocin.

Methods:

PEPTIDE EFFECTS ON ETHANOL TOLERANCE. To characterize peptide effects on maintenance of ethanol tolerance, mice were implanted with intracerebroventricular (icv) cannulae, and made tolerant to and physically dependent on ethanol by feeding them a liquid diet containing 7% ethanol, as previously described (Ritzmann and Tabakoff, J. Pharmacol. Exptl. Ther. 199: 158, 1976). Control mice were pair-fed a diet containing sucrose in amounts equicaloric to the ethanol. At 24 hours after withdrawal, animals were tested for tolerance, and then subdivided into groups that received once-daily icv injections of peptides or artificial CSF. Tolerance to the hypnotic effect of ethanol was tested at three-day intervals. To assess peptide effects on the development of ethanol tolerance, mice received daily icv injections of peptides during a three-day period of ingesting ethanol. Tolerance to the hypnotic effect of ethanol was tested at 24 hours after withdrawal.

VASOPRESSIN AND OXYTOCIN BIOSYNTHESIS. To study the effects of ethanol on vasopressin synthesis and release, a radioimmunoassay was used to measure vasopressin levels in rat posterior pituitary, plasma or cell culture medium (Dorsa and Bottemiller, Brain Res. 242: 151, 1982). RNA was isolated by previously-described methods, and mRNA was quantitated by slot blot and Northern blot techniques, using synthetic oligonucleotide probes labeled with <sup>32</sup>P (Chirgwin et al., Biochem. 18: 5294, 1979; Maniattis et al., Molecular Cloning, Cold Spring Harbor, 1982). Separation of AVP, oxytocin, and neurophysins was accomplished by an HPLC technique (White et al., Neurosci. 17: 133, 1986).

## Major Findings:

Our earlier studies with peptide analogs demonstrated particular structural requirements for neurohypophyseal peptide maintenance of ethanol tolerance, suggesting that peptide modulation of tolerance may be mediated by brain receptors which recognize AVP. Vasopressin receptors in peripheral organs have recently been classified as V1 or V2. Agonists at V2 receptors, found in kidney, stimulate adenylate cyclase activity, while agonist interaction with V1 receptors (liver and vascular smooth muscle) stimulate phosphatidylinositol (PI) metabolism and calcium mobilization. Ligand binding studies suggested that receptors in brain are similar to V1 receptors, and, in hippocampus, stimulation of PI metabolism by vasopressin has been reported. A number of analogs have been synthesized that act as selective agonists or antagonists at vasopressin receptors, and these compounds were used to characterize the receptors that mediate the effect of vasopressin on maintenance of ethanol tolerance. The ability of vasopressin to maintain tolerance to the hypnotic effect of ethanol was blocked more effectively by antagonists acting at V1 receptors than by a V2-selective antagonist. Similarly, a V1-selective agonist was more potent than AVP in maintaining tolerance, while V2-selective agonists were inactive. These data indicate that a brain receptor with the characteristics of a V1 receptor mediates the effect of vasopressin. If endogenous hormone is involved in maintaining tolerance to ethanol, administration of a vasopressin antagonist alone should facilitate the loss of tolerance. We found that a V1-selective antagonist, given alone, enhanced the rate of loss of tolerance, while a V2-selective antagonist did not, again supporting a role for a V1 receptor in the ability of vasopressin to maintain tolerance. In previous work, we showed that the maintenance of tolerance by vasopressin depended on the presence of intact noradrenergic systems in brain. Since, in the periphery, V1 receptors mediate peptide-induced increases in intracellular calcium, it is possible that AVP may interact with neuronally localized V1 receptors in brain to promote the release of norepinephrine and thus modulate tolerance. We have initiated ligand binding studies in membranes and by autoradiography to characterize mouse brain vasopressin receptors and to determine if they are localized presynaptically. Preliminary studies in membranes demonstrate high-affinity vasopressin binding only in the presence of nickel ion, which is thought to retard peptide metabolism, but which also appears to enhance specific binding. Very high nonspecific binding reduced the utility of a labeled V1 antagonist for assessing receptor characteristics in hippocampal membranes. Autoradiography may provide a more precise measure of peptide binding, and will give valuable information regarding localization of receptors.

In contrast to the maintenance of already-established tolerance by AVP, we found that vasopressin administered during the induction of tolerance blocked or delayed its development. Similar results were found when "environment-dependent" tolerance was studied. Recent studies showed that both V1 and V2 agonists and antagonists blocked the development of tolerance, and the receptor mediating this effect is therefore not clear (the antagonists are known to have partial agonist activities in some instances). Furthermore, other peptides, including oxytocin and analogs of the C-terminal portion of oxytocin, blocked the development of ethanol tolerance. These results are reminiscent of peptide effects on retrieval of memory (Flexner et al., Brain Res. 134: 139, 1977) and development of tolerance to morphine (Walter et al., Proc. Natl. Acad. Sci. USA 75: 4573, 1978). In the case of environment-dependent ethanol tolerance, it was suggested that vasopressin administration might interfere with the environmental cues that are

necessary for tolerance to develop. However, this explanation is less likely when animals are ingesting ethanol in a liquid diet. The fact that a variety of peptides are active suggests that they may interact with receptors different from the vasopressin receptor that mediates peptide maintenance of ethanol tolerance, and the mechanism of the block or delay of tolerance development needs further investigation.

If endogenous vasopressin is important for modulation of ethanol tolerance, one might expect to find that chronic ethanol ingestion alters the synthesis and release of AVP, either in the brain or in the periphery (or both). Acutely, ethanol is believed to inhibit AVP release, and it has been reported that circulating AVP levels are increased in alcoholics, although few results are available regarding AVP levels during chronic ethanol administration. We have developed the techniques for isolation and quantitation of hypothalamic vasopressin mRNA, as well as HPLC techniques that can be used to separate vasopressin, oxytocin and neurophysins. Levels of vasopressin in rat posterior pituitary and plasma have been determined, and are in the range of reported values. In a control experiment, rats were water-deprived for three days, and changes in posterior pituitary and plasma vasopressin and oxytocin were measured, as were hypothalamic vasopressin and oxytocin mRNA. The latter increased 2-fold while hypothalamic levels of vasopressin (but not oxytocin) decreased. Plasma vasopressin and oxytocin were increased in dehydrated rats. The data suggest that oxytocin and vasopressin synthesis and release are increased in dehydrated animals, and that vasopressin release overcomes synthesis, while this is not true for oxytocin. A Western blot method is being developed to quantitate neurophysins, which will provide more detailed information about rates of synthesis and release.

Recent studies have demonstrated the presence of immunoreactive oxytocin and vasopressin in extrahypothalamic tissues, including the anterior pituitary, testis, ovary and adrenal gland. We have identified mRNA for these peptides (using synthetic oligonucleotide probes) in anterior pituitary, testis and in a mouse-derived Leydig tumor cell line (MLTC-1), and have demonstrated by radioimmunoassay that the tumor cell culture produces vasopressin. In dehydrated rats, vasopressin and oxytocin mRNAs were increased in anterior pituitary and testis, as in the hypothalamus. Acute treatment (24 hours) of MLTC-1 cells with ethanol decreased vasopressin mRNA levels. We have also found that treatment of MLTC-1 cells with human chorionic gonadotropin or cyclic AMP decreased vasopressin and oxytocin mRNA levels, suggesting that these compounds may be physiological regulators of Leydig cell AVP synthesis. Thus, we are now in a position to investigate the effects of chronic ethanol exposure on several aspects of hypothalamic and extrahypothalamic vasopressin and oxytocin synthesis and release. Changes in plasma hormone levels produced by ethanol may reflect changes in release from peripheral organs as well as from the posterior pituitary, and may contribute to observed effects on ethanol tolerance.

#### Significance to Biomedical Research and the Program of the Institute:

The development of tolerance to ethanol allows the intake of large amounts of ethanol by an individual, possibly leading to development of physical dependence and to pathological changes in the CNS and peripheral organs. The finding that vasopressin, a naturally-occurring hormone, as well as vasopressin antagonists, can modulate tolerance, provides the opportunity for development of benign therapies to modify the development and maintenance of ethanol. From a

theoretical standpoint, our studies provide support for the hypothesis that tolerance and learning, or memory, as CNS adaptive mechanisms, share certain underlying mechanisms. These studies also offer a means both to analyze the biochemical mechanism of action of neurohypophyseal hormones in the CNS, as related to their behavioral effects, and to understand the neurochemical basis of tolerance, as well as learning and memory. Furthermore, these investigations enhance our understanding of the regulation of neurohypophyseal hormone synthesis and release, and the effect of ethanol on this regulation. The mechanisms that regulate extrahypothalamic vasopressin and oxytocin biosynthesis and release, and the possible roles of these hormones in development or maintenance of ethanol tolerance, will be elucidated in our studies.

#### Proposed Course:

Studies on the binding of AVP and specific antagonists in brain membranes will be performed by autoradiography, and analysis of peptide effects on adenylate cyclase activity, polyphosphoinositide metabolism and neurotransmitter release in brain will be initiated. Effects of acute and chronic ethanol treatment on hypothalamic AVP and oxytocin mRNA, neurophysin, and levels of AVP in plasma and posterior pituitary will be studied. The effects of ethanol on the synthesis and release of extrahypothalamic AVP, oxytocin and dynorphin, and the physiological mechanisms regulating release of these hormones, will be investigated.

#### Publications:

1. Mannix, S.A., Hoffman, P.L. and Melchior, C.L.: Intraventricular arginine vasopressin blocks the acquisition of ethanol tolerance in mice. Eur. J. Pharmacol., 128: 137, 1986.
2. Tabakoff, B., Cornell, N. and Hoffman, P.L.: Alcohol tolerance. Ann. Emerg. Med. 15: 1005-1012, 1986.
3. Tabakoff, B. and Hoffman, P.L.: Tolerance and the etiology of alcoholism: Hypothesis and mechanisms. Alcoholism: Clin. Exptl. Res., in press.
4. Hoffman, P.L., Szabo, G. and Tabakoff, B.: The effects of vasopressin and related peptides on tolerance to ethanol. Proc. Intl. Symp. on Peptide and Amino Acid Transport, in press.
5. Hoffman, P.L., Szabo, G. and Tabakoff, B.: Vasopressin and alcohol tolerance. Substance Abuse, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00705-01 LPPS

## PERIOD COVERED

October 1, 1986 - September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## In Vitro Models for Ethanol Effects on Receptor-Mediated Processes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. Hoffman	Section Chief	LPPS, NIAAA
	B. Tabakoff	Acting Chief	LPPS, NIAAA
Others:	C. Chung	Staff Fellow	LPPS, NIAAA
	C. Rabe	Senior Staff Fellow	LPPS, NIAAA
	P. Rathna Giri	Visiting Associate	LPPS, NIAAA
	Y. Watanabe	Visiting Fellow	LPPS, NIAAA
	S. Culp	Physical Sci. Tech.	LPPS, NIAAA
	F. Moses	Guest Researcher	LPPS, NIAAA

## COOPERATING UNITS (if any)

CPB, NIMH (L. Tamarkin)

## LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

## SECTION

Section on Receptor Mechanisms

## INSTITUTE AND LOCATION

NIAAA, 12501 Washington Ave., Rockville, MD 20852

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

2.75

## OTHER:

1.25

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
 ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unadduced type. Do not exceed the space provided.)

A major focus of our work involves an evaluation of the acute and chronic effects of ethanol in the CNS. However, the brain represents a heterogeneous collection of cell types, and distinction of direct and indirect effects of ethanol can be difficult. We are developing in vitro organ and tissue culture systems that can be used to monitor specific, direct effects of ethanol, for comparison and contrast with results obtained in brain tissue and in vivo. We have developed a primary culture of cerebellar granule cells in which beta-adrenergic receptors and agonist-stimulated cyclic AMP production can be measured; the role of glial and neuronal elements in these responses is being analyzed. Stimulation of cyclic GMP production in cerebellar granule cells is also being determined. Ethanol appears to inhibit this stimulation, similar to results reported in brain. Ethanol enhances isoproterenol-stimulated cyclic AMP and melatonin production by pineal glands in culture. These findings of enhanced receptor-effector coupling are similar to what we have reported in certain brain areas, and allow an investigation of the effects of ethanol on the physiological consequences of receptor activation. Ethanol also appears to enhance cyclic AMP production in pheochromocytoma (PC12) cells, and these cells are being characterized with respect to stimulation of cyclic AMP production by vasoactive intestinal peptide, adenosine and forskolin. In PC12 cells, we have also found that low concentrations of ethanol (25 mM) inhibit muscarine- and depolarization-induced norepinephrine release. In the former case, ethanol appears to modulate muscarine-stimulated inositol trisphosphate production, and therefore calcium mobilization. These systems allow for detailed examination of the sites and mechanisms of action of ethanol and of changes in biochemical systems that may, in vivo, be associated with ethanol tolerance and physical dependence.

## Project Description

### Investigators:

P. Hoffman	Section Chief	LPPS, NIAAA
B. Tabakoff	Acting Chief	LPPS, NIAAA
C. Rabe	Senior Staff Fellow	LPPS, NIAAA
P. Rathna Giri	Visiting Associate	LPPS, NIAAA
Y. Watanabe	Visiting Fellow	LPPS, NIAAA
S. Culp	Physical Sci. Tech.	LPPS, NIAAA
F. Moses	Guest Researcher	LPPS, NIAAA
C. Chung	Staff Fellow	LPPS, NIAAA
L. Tamarkin	Research Biologist	CPB, NIMH

### Objectives:

The goal of this project is to develop in vitro systems that can be used to model certain aspects of nervous system function, particularly with respect to the function of neurotransmitter and neuromodulator receptors. In vitro cell and organ cultures provide relatively homogeneous systems in which the biochemical as well as physiological responses to receptor activation can be studied in detail in intact cells. These preparations can also be used to evaluate the acute and chronic effects of ethanol on specific receptor-mediated processes under controlled conditions, in systems that mimic the intact physiology of the CNS. Thus, these studies are designed for comparison with studies in brain, to define the specific sites of action of ethanol, and to determine if adaptive changes in various aspects of receptor-effector coupling processes occur at the initial sites of action of ethanol. The findings from these studies can be used to devise experimental protocols that will, in vivo, relate biochemical changes to the development of ethanol tolerance and/or dependence.

### Methods:

**CELL CULTURES.** The rat pheochromocytoma cell line, PC12, was grown in plastic tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) containing heat-inactivated horse serum and fetal bovine serum at 37° in a humidified atmosphere of 90% air: 10% CO<sub>2</sub>. Cell viability was assessed by trypan blue exclusion. Cerebellar neuronal cultures were obtained from eight-day old rats (Wilkin et al., Brain Res. 115: 181, 1976). Cells were plated onto Falcon dishes coated with poly-L-lysine, and were grown in basal modified Eagle's medium containing heat-inactivated fetal calf serum, gentamycin and KCl. The culture dishes were incubated at 37° in humidified 95% air:5% CO<sub>2</sub>. After 18-20 hr, cytosine arabinoside was added to the culture medium to inhibit the replication of non-neuronal cells. The culture medium was renewed at days 2, 5 and 8 of culture, and cells were used on day 8 of culture. Cell viability was monitored with trypan blue exclusion and by 51 Cr release. Glial cells were identified by immunofluorescence, using antiserum to glial fibrillary acidic protein.

**NEUROCHEMICAL MEASUREMENTS IN CULTURED CELLS.** In PC12 cells, the release of 3H-norepinephrine (NE) was measured in a continuous superfusion system. Cells were incubated with 3H-NE, washed, and fractions of superfusate were collected. Ligand binding to cholinergic receptors was assayed using 3H-N-methylscopol-



Cerebellar granule cell cultures are also being used to evaluate the effects of ethanol on another second messenger system, i.e., agonist-stimulated cyclic GMP production. In brain, ethanol has been consistently shown to reduce the levels of cerebellar cyclic GMP, but the mechanism of this action is not clear. It has been postulated that arachidonic acid metabolites may play a role in receptor-mediated stimulation of guanylate cyclase activity, and, since ethanol has been reported to alter arachidonate metabolism, it could affect cyclic GMP levels via this pathway. To characterize the action of ethanol, the effects of ethanol on stimulation of cyclic GMP production by glutamate and atrial natriuretic peptide (ANP) were studied in the granule cells. Two isomers of ANP (ANP II and ANP III) stimulated cyclic GMP production in a dose-dependent manner. Stimulation by ANP II was inhibited by 50-100 mM ethanol, while ethanol had less effect on stimulation by ANP III. However, ANP III stimulation of cyclic GMP production was inhibited by propanol and butanol. The effect of ethanol on stimulation of cyclic GMP production by glutamate has not yet been evaluated. It has been reported (Paul et al., Science 235: 1224, 1987), that the receptor for ANP and guanylate cyclase exist as components of a single protein in rat adrenocortical carcinoma cells. If this is also true in rat cerebellar granule cells, differential or similar effects of ethanol on ANP- and glutamate-stimulated cyclic GMP production could provide insight into both the mechanism of action of ethanol, and of the hormones that enhance cyclic GMP production.

PC12 cells secrete catecholamines in response to a number of stimuli, and serve as a model system for investigations of neuronal secretion mechanisms. We have characterized muscarine-stimulated release of norepinephrine (NE) in these cells, and have found that ethanol, at concentrations as low as 25 mM, inhibits this secretion. Inhibition of secretion was positively correlated with the ability of ethanol to inhibit muscarine-stimulated increases in intracellular free calcium. Ethanol did not affect muscarine binding to the cells. Muscarine-induced increases in intracellular free calcium and NE release are mediated by increases in phosphatidylinositol metabolism, leading to production of inositol trisphosphate. By using an HPLC method that separates various inositol phosphate isomers, we have found, in initial studies, that ethanol may reduce the concentration of an isomer responsible for mobilization of intracellular calcium. Higher concentrations of ethanol stimulates basal NE release and increases intracellular calcium levels, but these effects are independent of the inhibition of muscarine-induced responses. Ethanol also inhibited depolarization-induced release of NE, which is dependent on influx of extracellular calcium.

PC12 cells are also being used to further evaluate the effects of ethanol on receptor-stimulated adenylate cyclase (AC) activity. Vasoactive intestinal peptide (VIP), adenosine and forskolin stimulate cyclic AMP production in PC12 cells, and preliminary results suggest that ethanol enhances basal AC activity in these cells. The cells may therefore serve as model systems for the effects of acute and chronic ethanol treatment in the brain, and will allow a more precise determination of the direct effects of ethanol, as well as its site and mechanism of action.

#### Significance to Biomedical Research and the Program of the Institute:

Using intact cells and organs in culture, we have found many of the same effects of ethanol on neurotransmitter receptor-effector coupling processes

amine and cells that had been replated onto poly-L-lysine-coated dishes. After the incubation with ligand, cells were washed and solubilized with NaOH, and bound radioactivity and protein were quantitated. Intracellular free calcium was measured with the fluorescent indicator, Quin 2. Phosphatidylinositol metabolism was assayed by preincubating cells with  $^3\text{H}$ -myo-inositol and measuring accumulation of labelled inositol phosphates in the presence of lithium. Inositol phosphates were separated by conventional column chromatography (Berridge et al., *Biochem. J.* 212: 473, 1983) or by HPLC techniques (Morgan et al., *J. Biol. Chem.* 262: 1166, 1987). Radioimmunoassays of cyclic AMP and cyclic GMP were performed with kits from New England Nuclear Corp. (Boston, MA).

**PINEAL ORGAN CULTURES.** Pineal glands were obtained from male Sprague-Dawley rats, transferred onto sterile nylon mesh supports, and placed in wells of tissue culture plates containing BGJ6 standard culture medium with ascorbate, glutamine and antibiotics. Glands were incubated at  $37^{\circ}$  for 24 hours before experiments. Melatonin and cyclic AMP released into the medium were quantitated by radioimmunoassay.

#### Major Findings:

In the pineal gland, norepinephrine stimulation of adenylate cyclase activity leads to the synthesis and release of melatonin. Therefore, this gland provides a model in which the physiological consequences of agonist interaction with the beta-adrenergic receptor can be readily assessed. We found that low concentrations of ethanol enhanced isoproterenol-stimulated cAMP production, similar to results in brain tissue, and ethanol also increased isoproterenol-stimulated melatonin production. Other alcohols were more potent than ethanol in this system, indicating that the enhanced response to isoproterenol is not simply an osmotic effect. Ethanol alone did not alter either cyclic AMP or melatonin production, consistent with the postulate that ethanol alters the interaction of isoproterenol with the pineal beta-adrenergic receptor or, as in brain, may interact with the proteins involved in the receptor-effector coupling process.

Primary cultures of cerebellar granule cells were also tested as possible model systems in which to evaluate beta-adrenergic receptor function. In these cultures, isoproterenol was found to stimulate cyclic AMP production in a dose-dependent manner, and this action of isoproterenol was blocked by beta-adrenergic antagonists. The antagonist 125I-iodocyanopindolol (ICYP) was used to characterize beta-adrenergic ligand binding sites in the cell membranes, and the results indicated the presence of beta-adrenergic receptors in the cerebellar granule cell cultures. However, these cultures are contaminated with glial cells (about 5%), and studies are underway to localize the beta-adrenergic receptors to neuronal or glial cells. One method is to permit the growth of glial cells to enrich the culture with these cells, and to determine the change in beta-adrenergic receptor number. The effects of ethanol on the function of these receptors will be evaluated, regardless of whether they are glial or neuronal in origin, for comparison to effects in brain cerebellar membranes. The primary cultures provide a homogeneous model in which changes in the characteristics of proteins such as Gs can be studied.

that we and others have reported in brain. For example, ethanol enhances isoproterenol stimulation of cyclic AMP production in pineal, and stimulates basal AC activity in PC12 cells. The PC12 cells appear to be more sensitive to the effect of ethanol on cholinergic responses than brain slices (see Hoffman et al., Mol. Pharmacol. 30: 13, 1986), and allow measurement of the physiological consequences of cholinergic receptor activation. The in vitro systems are relatively homogeneous, and can be used to isolate proteins (e.g., G proteins) that appear to be involved in the actions of ethanol in order to evaluate changes at the molecular level. These studies provide data regarding the site and mechanism of action of ethanol that can be extrapolated to in vivo systems, and thus further our understanding of certain aspects of the acute and chronic effects of ethanol in the CNS.

#### Proposed Course:

Primary cultures of cerebellar granule cells will be further characterized with respect to activation of cyclic AMP and cyclic GMP production and the effects of ethanol on the second messenger systems. The site and mechanism of action of ethanol will be investigated. The acute and chronic effects of ethanol on receptor-stimulated AC systems in pineal, granule cells, PC12 cells and other transformed cell lines will be studied. These investigations will provide evidence for the specific or general nature of ethanol's effects, and the chronic studies will allow a determination of the nature of adaptive changes in response to ethanol. The various cell lines will be used, if appropriate, for isolation, quantitation and characterization of proteins (such as Gs) that may mediate the acute effects of ethanol and whose function may be altered during chronic ethanol exposure.

#### Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00464-06 LPPS
PERIOD COVERED October 1, 1986 - September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Ethanol and Cellular Calcium Metabolism		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) P.I.: H. C. Pant Research Chemist LPPS, NIAAA  Others: J. Shah Visiting Fellow LPPS, NIAAA M. Virmani Research Chemist LPPS, NIAAA		
COOPERATING UNITS (if any) Laboratory of Molecular Biology, Univ. of Wisconsin, Madison, Wisconsin (R. Silver)		
LAB/BRANCH Laboratory of Physiologic and Pharmacologic Studies		
SECTION Section on Receptor Mechanisms		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Ave., Rockville, MD 20852		
TOTAL MAN-YEARS: 1.9	PROFESSIONAL: 0.9	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Recent studies show that several cell types that mobilize intracellular calcium in response to hormones or neurotransmitters also hydrolyze phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and inositol trisphosphate (IP3), which may mediate the mobilization of intracellular calcium. In the present study, we investigated the involvement of IP3 in calcium mobilization in rat brain microsomes. Ethanol-induced neurotransmitter release in unstimulated synaptosomes was found to be independent of the extrasynaptosomal calcium concentration. Therefore, the effects of alcohol on microsomal calcium uptake and release were also studied. IP3 caused a rapid release of calcium from brain microsomes. <u>In vitro</u> addition of 100 mM ethanol had no effect on ATP-dependent calcium accumulation in the microsomes, but the same concentration of ethanol released 25% of the total accumulated calcium from the microsomes. Ethanol induced calcium release in a concentration-dependent manner over the range of 30 mM to 500 mM. The amount of calcium release increased with higher alcohols. The effect of ethanol was temperature-dependent, suggesting a diffusion-controlled process for calcium transport. These results indicate that the stimulatory effect of ethanol on resting release of neurotransmitters in rat brain may be due to the microsomal release of calcium.		

Project DescriptionInvestigators:

H. C. Pant	Research Chemist	LPPS, NIAAA
J. Shah	Visiting Fellow	LPPS, NIAAA
M. Virmani	Research Chemist	LPPS, NIAAA

Objectives:

The mobilization of calcium ion from intracellular stores in response to hormones or neurotransmitters plays an important role in the regulation of various cellular processes. Recent studies show that cell surface receptors that mobilize intracellular calcium and, thereby, increase cytoplasmic free calcium, also hydrolyze phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and inositol trisphosphate (IP<sub>3</sub>). IP<sub>3</sub> is believed to serve as a second messenger to stimulate the release of calcium from internal stores. In order to test the possible involvement of IP<sub>3</sub> in calcium release in the brain, we studied the effects of IP<sub>3</sub> in a brain microsomal fraction and investigated the mechanism of IP<sub>3</sub>-induced calcium release. In addition, the effect of ethanol on these processes has been investigated.

Methods:

Brain microsomes were prepared by differential ultracentrifugation as described by Edelman et al. (J. Neurosci. 5: 2609, 1985) and purity was checked by electron microscopy. Calcium and potassium fluxes were determined by measuring the radioactive calcium and rubidium, respectively, trapped in the microsomes.

To measure neurotransmitter release, a synaptosomal preparation was loaded with 3H-norepinephrine (NE) and was added to Krebs-Ringer buffer containing CaCl<sub>2</sub> or EGTA (no calcium). Transmitter release was initiated with the addition of 50 mM KCl (+ calcium) or 5  $\mu$ M A23187 (- calcium), and release was studied in the presence and absence of ethanol. The reaction mixture was incubated for 5 minutes at 37°C and the reaction was terminated by centrifugation at 4°C. Released radioactivity was determined by liquid scintillation counting.

Major Findings:

ATP-dependent accumulation of calcium was observed in brain microsomes and was inhibited by sodium vanadate but was unaffected by calcium channel blockers, including ruthenium red, verapamil or nifedipine. IP<sub>3</sub> caused a rapid release of calcium from the microsomal preparation which was followed by a slow re-uptake. IP<sub>3</sub> released calcium in a concentration-dependent manner and release was maximal at a concentration of 0.2  $\mu$ M. IP<sub>3</sub>-induced calcium release was dependent on free extramicrosomal calcium, also required the presence of potassium chloride (100 mM). Tetraethylammonium chloride (TEA) and 9-TEA blocked IP<sub>3</sub>-induced calcium release. Since the efflux of calcium induced by IP<sub>3</sub> is associated with an influx of potassium, modulation of the potassium channel by TEA or 9-TEA may account for the inhibition of IP<sub>3</sub>-induced calcium release.

Ethanol stimulated norepinephrine (NE) release from synaptosomes in a dose-dependent manner. A statistically significant effect was found at 30 mM ethanol. Ethanol-induced NE release was unaffected by the removal of

extrasynaptosomal  $\text{Ca}^{2+}$ , indicating that ethanol may release intracellular calcium, and thus elevate cytosolic calcium. In vitro addition of ethanol (100 mM) had no effect on ATP-dependent calcium accumulation in microsomes. However, the same concentration of ethanol released 25% of total accumulated calcium from the microsomes. Ethanol induced calcium release in a concentration-dependent manner over the range of 30 mM to 500 mM. The effect of alcohol was temperature-dependent, suggesting a diffusion-controlled process for calcium transport. These results indicate that the stimulatory effect of ethanol on resting release of neurotransmitters from synaptosomes may be due to the microsomal release of calcium.

#### Significance to Biomedical Research and the Program of the Institute:

The role of calcium as an activator and regulator of many biological processes has been well recognized. An increase in cytoplasmic calcium concentration underlies such important biological processes as secretion of neurotransmitters and hormones, regulation of enzyme activities, control of membrane ion permeabilities and muscle contraction. There are a number of cellular mechanisms which control cytosolic calcium levels. The characterization of inositol trisphosphate release of calcium from brain microsomes extends our understanding of the regulation of brain calcium metabolism. In addition, the finding that ethanol alters brain microsomal calcium release provides evidence for an important mechanism by which ethanol can affect neuronal activity in the CNS.

#### Proposed Course:

The effects of acute and chronic ethanol exposure on agonist and IP<sub>3</sub>-induced mobilization of calcium in rat brain will be further characterized.

#### Publications:

Shah, J., Cohen, R.S. and Pant, H.C.: IP<sub>3</sub> induced calcium release in brain microsomes. Brain Res., in press.





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER <div style="text-align: center; font-weight: bold;">Z01 AA 00704-02 LPPS</div>
PERIOD COVERED October 1, 1986 - September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Genetic Variation, Linkage Relationships and Regulation of ADH		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: M. Smith  Others: B. Tabakoff L. Liu	Visiting Scientist  Acting Chief Guest Researcher	LPPS, NIAAA  LPPS, NIAAA LPPS, NIAAA
COOPERATING UNITS (if any) Univ. of Iowa, (J. Murray)		
LAB/BRANCH Laboratory of Physiologic and Pharmacologic Studies		
SECTION Section on Receptor Mechanisms		
INSTITUTE AND LOCATION NIAAA, 12501 Washington Ave., Rockville, MD 20852		
TOTAL MAN-YEARS: <div style="text-align: center;">1.2</div>	PROFESSIONAL: <div style="text-align: center;">0.2</div>	OTHER: <div style="text-align: center;">1.4</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (b) Human tissues         </div> <div style="width: 30%;"> <input type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Ethanol is metabolized by two enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). cDNA probes and genomic DNA probes have been utilized to investigate genetic variation at the ADH and ALDH loci, the mapping relationships of ADH genes, factors involved in regulation of ADH gene expression in different tissues and during development, and the altered expression of ADH in liver tumors. Results of these studies have led to the identification of population differences in the frequencies of ADH DNA polymorphisms. Studies have also been carried out to define whether specific ADH DNA polymorphisms are in linkage disequilibrium with ADH kinetic variations. The map relationships of ADH and 12 other markers on human chromosome 4 have been established. A study of ADH mRNA in adult liver, fetal liver, intestine and lung, and analysis of sequence data revealed that for a particular ADH gene the polyadenylation site which is predominantly used varies in different tissues and may vary in development. Analysis of ADH3 in hepatomas revealed allele loss in three out of five hepatomas. DNA alterations at the epidermal growth factor locus (known to be linked to ADH3) were also observed in three tumors, suggesting that structural changes in the 4q21-4q27 region occur in a significant proportion of hepatomas.</p> <p>This project has been terminated.</p>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00400-02 LPPS												
PERIOD COVERED October 1, 1986 - September 30, 1987														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Selective Breeding for Ethanol Tolerance														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: B. Tabakoff</td> <td style="width: 33%;">Acting Chief</td> <td style="width: 33%;">LPPS, NIAAA</td> </tr> <tr> <td>R. M. Werner</td> <td>Veterinarian</td> <td>DICBR, NIAAA</td> </tr> <tr> <td colspan="3"> </td> </tr> <tr> <td>Other: P. L. Hoffman</td> <td>Section Chief</td> <td>LPPS, NIAAA</td> </tr> </table>			PI: B. Tabakoff	Acting Chief	LPPS, NIAAA	R. M. Werner	Veterinarian	DICBR, NIAAA				Other: P. L. Hoffman	Section Chief	LPPS, NIAAA
PI: B. Tabakoff	Acting Chief	LPPS, NIAAA												
R. M. Werner	Veterinarian	DICBR, NIAAA												
Other: P. L. Hoffman	Section Chief	LPPS, NIAAA												
COOPERATING UNITS (if any) VRB, SAS, (C. Hansen, N. Jackson, W. Watson)														
LAB/BRANCH Laboratory of Physiologic and Pharmacologic Studies														
SECTION Section on Receptor Mechanisms														
INSTITUTE AND LOCATION NIAAA, 12501 Washington Ave., Rockville, MD 20852														
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0												
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input type="checkbox"/> (b) Human tissues</td> <td><input checked="" type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews					
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither												
<input type="checkbox"/> (a1) Minors														
<input type="checkbox"/> (a2) Interviews														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>           This project is designed to produce lines of rats selected for high and low degrees of tolerance to ethanol. Animals inhale ethanol chronically in specially-constructed chambers, and are then tested for functional tolerance to the hypnotic effect of ethanol. Initial studies have confirmed genetic heterogeneity with respect to the development of tolerance in the N:NIH rats that will serve as breeding stock. Animals demonstrating high or low degrees of tolerance will be mated, with precautions taken to minimize inbreeding. At the limit of selection, animals selected for each trait will theoretically contain all alleles associated with that trait, while alleles not associated with the trait will be randomly distributed. Therefore, these animals will be a resource for all researchers interested in the biochemical determinants of alcohol tolerance.         </p>														

Project Description

B. Tabakoff	Acting Chief	LPPS, NIAAA
P.L. Hoffman	Section Chief	LPPS, NIAAA
R. M. Werner	Veterinarian	DICBR, NIAAA
N. Watson	Chief	VRB, SAS
W. Jackson	Deputy Chief	VRB, SAS
C. Hansen	Geneticist	VRB, SAS

Objectives:

The goal of this project is to breed selected lines of rats that develop high and low degrees of functional tolerance to ethanol. Theoretically, at the limits of selection, the selection process will result in animals in which all of the alleles, present in the initial gene pool, that are related to a high degree of tolerance, will be represented in animals of the "high tolerance" line. Those alleles related to a low degree of tolerance will all be represented in the "low-tolerance" line. On the other hand, alleles unrelated to the selected phenotype will not be subjected to directional influences. Therefore, biochemical traits that differ between the selected lines will be related to the ability to develop tolerance to ethanol. The availability of these animals will allow investigators to definitively study the neurochemistry of tolerance.

Methods:

The breeding stock of rats will be the heterogeneous N:NIH stock. We have previously tested these animals for development of tolerance to the hypnotic effect of ethanol, and found sufficient variability to warrant using the animals to produce selected lines (Tabakoff and Culp, Alc: Clin. Exptl. Res. 8: 495, 1984). Rats are exposed to ethanol by inhalation in a chamber for eight days, and tolerance to the hypnotic effect of ethanol is tested by measuring blood ethanol levels at the regain of the aerial righting reflex, following injection of 2.5 g/kg of ethanol (Tabakoff and Culp, 1984). Our previous studies showed that metabolic tolerance does not develop with the procedure used. Animals demonstrating a high or low degree of tolerance will be mated, using procedures that minimize inbreeding.

Major Findings:

To confirm initial results regarding genetic variability with respect to tolerance development, we have screened one male and one female offspring from each of 60 breeding pairs of N:NIH rats. The results confirm a high degree of heterogeneity in ethanol tolerance development in these rats. Blood ethanol levels at the regain of the aerial righting reflex following a dose of 2.5g/kg of ethanol were measured before animals were placed in the ethanol inhalation chambers, and after eight days in the chambers, during which time blood ethanol levels were increased in a stepwise manner to a maximal level of 325-350 mg%. The change in blood ethanol levels at regain of the righting reflex following chronic ethanol inhalation (a significant increase reflects the development of functional ethanol tolerance) ranged from -80% to 200%. There were no apparent

overall differences in the responses of male and female rats. Forty-seven percent of the animals had more than a 50% increase in the blood ethanol level at the regain of the aerial righting reflex, and this change has been selected as the threshold criterion for choosing animals with a high degree of tolerance. Animals showing no change, or a decrease in blood ethanol level at regain of the righting reflex (increased sensitivity), will be mated to produce lines of animals that develop a low degree of tolerance to ethanol.

#### Significance to Biomedical Research and the Program of the Institute:

The development of tolerance to ethanol allows the intake of large amounts of ethanol by an individual, possibly leading to development of physical dependence and to pathological changes in the CNS and peripheral organs. The development of lines of animals with differing ability to develop ethanol tolerance will provide a model in which definitive determinations of the neurochemical basis for ethanol tolerance can be made. These animals will provide a resource for all investigators interested in the determinants of ethanol tolerance. Understanding the biochemical basis for functional tolerance can eventually lead to therapies that can modify the development or extent of tolerance.

#### Proposed Course:

In further preliminary studies of genetic heterogeneity, the rate and extent of tolerance development in the inbred strains of rats used to produce the N:NIH stock will be confirmed. Selective breeding of the lines of rats with high and low degrees of functional ethanol tolerance will be initiated.

#### Publications:

None.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00701-03 LPPS

## PERIOD COVERED

~~October 1, 1986 - September 30, 1987~~

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

~~Ethanol and the GABA-BDZ-Barbiturate Receptor/Chloride Ionophore~~

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Tabakoff Acting Chief LPPS, NIAAA  
S. Liljequist Visiting Associate LPPS, NIAAA

Others: S. Culp Phys. Sci. Tech. LPPS, NIAAA  
Y. Watanabe Visiting Fellow LPPS, NIAAA

## COOPERATING UNITS (If any)

Georgetown University, (E. Costa, A. Guidotti);  
NIMH, CNB, (S. Paul, P. Suzdak)

## LAB/BRANCH

~~Laboratory of Physiologic and Pharmacologic Studies~~

## SECTION

~~Section on Receptor Mechanisms~~

## INSTITUTE AND LOCATION

~~NIAAA, 12501 Washington Ave, Rockville, MD 20852~~

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

~~2.5~~~~1.5~~~~1.0~~

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Many pharmacological and physiological effects of ethanol have been postulated to be mediated via GABA-containing neuronal systems in the CNS. The GABA receptor exists as part of a complex containing receptors for benzodiazepines (BDZ), barbiturates and the chloride ion channel. To examine the effects of ethanol on this complex, we have studied binding properties of 35-S-t-butylbicyclophosphorothionate (35-S-TBPS), a ligand which interacts with the regulatory site of the chloride ion channel, in membranes of cortex and cerebellum of C57Bl mice in the presence and absence of ethanol. In the presence of bromide ion *in vitro*, ethanol produced a dose-dependent inhibition of TBPS binding, but did not potentiate inhibition of TBPS binding by GABA or pentobarbital. In the presence of chloride ion, ethanol was less potent at inhibiting TBPS binding. However, under these conditions, GABA and pentobarbital had biphasic effects on TBPS binding in cortex, and low concentrations of ethanol reduced the stimulatory effects of these compounds on TBPS binding. This project has been incorporated into Project Z01 AA 00702-03 LPPS.





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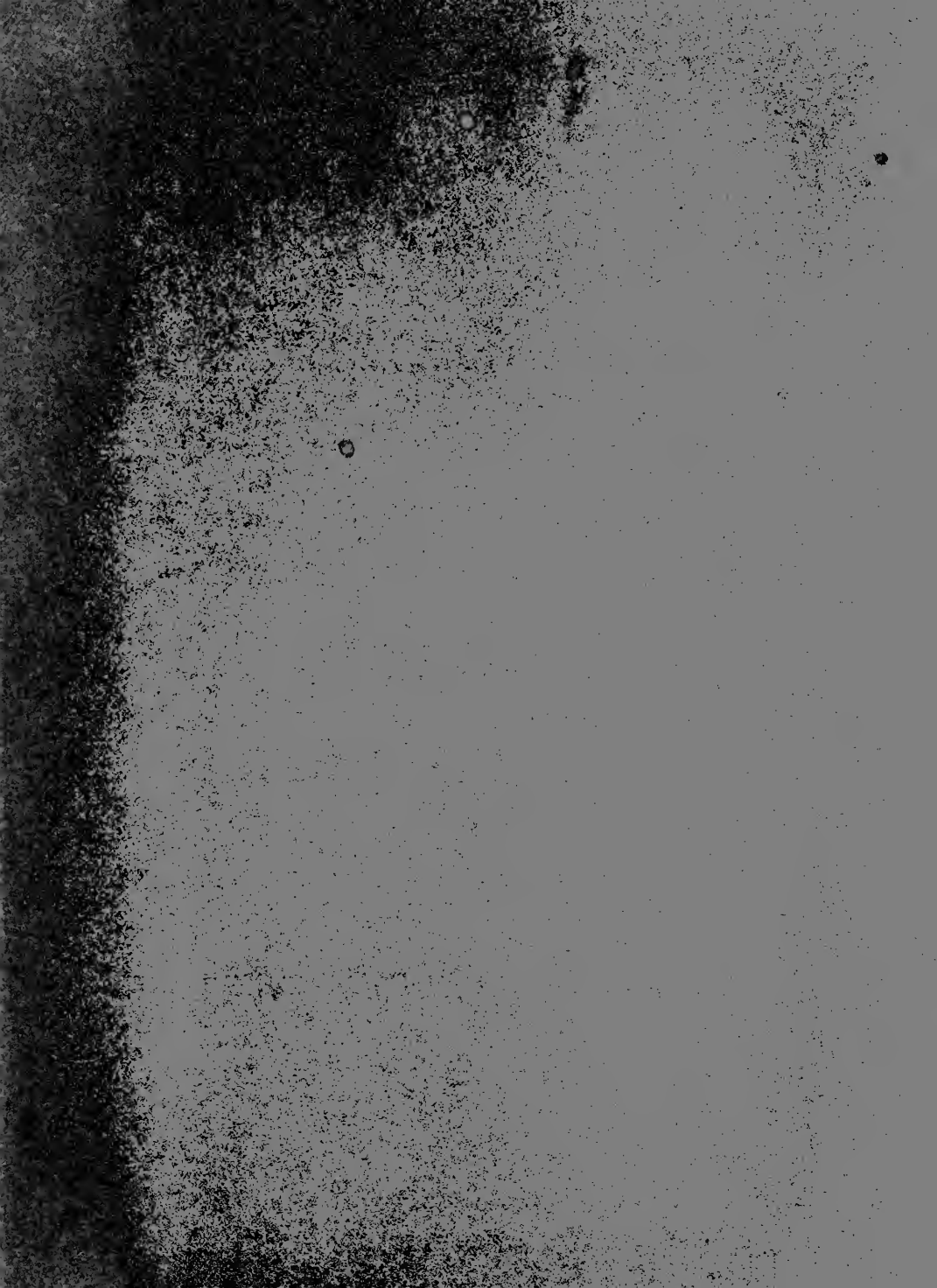
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